The modulating effect of myo-inositol and other antidepressants on the mRNA levels and protein expression of selected subcellular enzymes

MARINA VAN ROOYEN

(B.Pharm)

Dissertation submitted for the degree Magister Scientiae in Pharmacology at the North-West University (Potchefstroom Campus)

Supervisor:	Prof. C.B. Brink
Co-supervisor:	Prof. P. Pretorius
Assistant supervisor:	Prof B.H. Harvey

2005

Potchefstroom

Acknowledgements

I gratefully and humbly extend my sincere gratitude to the Almighty Father, the most important person in my life, for giving me the strength and perseverance to finish this study. I wish to express my sincere gratitude to the many people who have contributed to this dissertation. The following persons, however, deserve special mention and are acknowledged for their assistance:

- Prof. C. B. Brink, in his capacity as supervisor, my appreciation for his excellent guidance, advice and encouragement.
- Prof. P. Pretorius, in his capacity as co-supervisor, and Prof. B. H. Harvey, in his capacity as assistant supervisor, my appreciation for their willingness to assist and their expert guidance.
- The department of Pharmacology for financial and technical support.
- Schaun and Ané Korff for their advice and technical assistance.
- Carla Martin-Furness for her advice.
- Sharlene Nieuwoudt and Maureen Steyn for your friendship, encouragement and assistance.
- My fellow M-students, Izelle, Renché, Kenny, Zakkiyya, Bien and Tanya, for their assistance and encouragement.
- My parents and family for their constant love and support.
- To my fiancée, Renier, for all his understanding, patience and love.

Abstract

***Title:* The modulating effect of myo-inositol and other antidepressants on the mRNA levels and protein expression of selected subcellular enzymes**

myo-Inositol (*m*Ins), a natural component of the human diet and essential precursor of several signalling pathways, including that of G protein-coupled receptors, has also been shown to be effective in the treatment of psychiatric disorders such as depression, obsessive compulsive disorder and panic disorder. Most likely since *m*Ins is a simple isomer of glucose, no serious side effects have been reported with its use, even at high oral doses of *m*Ins. Previous studies suggest that the therapeutic action of *m*Ins may include reduced serotonin $5HT_{2A}$ and muscarinic acetylcholine receptor function. An important signal transduction system that may possibly be involved in the mechanism of action of antidepressants is phosphoinositide (PI) turnover. In this signalling system PI-phospholipase C (PLCβ1), that is implicated in the in the mechanism of action of antidepressants and anxiolytics, is activated.

The mechanism of action of *m*Ins, however, still remains elusive and needs further investigation. In this study a possible modulatory role of 24-hour pre-treatment of human neuroblastoma cell line (SH-SY5Y) with *m*Ins on mRNA levels and protein expression of phospholipase C-β1 (PLCβ1) and glycogen synthase kinase 3β (GSK3β) was investigated. The effects of *m*Ins were also compared to that of other prototype antidepressants, such as fluoxetine (a selective serotonin reuptake inhibitor), imipramine (a tricyclic antidepressant), lithium and another drug with potential antidepressant effects, sildenafil (phosphodiesterase 5-type (PDE5) inhibitor). Real-time reverse transcription Polymerase Chain Reaction (RT-PCR) was performed in order to investigate the mRNA levels, while protein expression in membranes and the cytosol fraction of cells were quantified with Western blots.

The expression of PLCβ1 was decreased after pre-treatments with imipramine or *myo*-inositol in combination with fluoxetine. In addition, sildenafil alone or in combination with *myo*-inositol, also decreased the expression of membrane-bound PLCβ1. However, a 24-hour pre-treatment with lithium did not alter PLCβ1 expression significantly. Determined mRNA levels for the expression of PLCβ1 were consistent in these findings, except for the inhibition of the mRNA for the expression of PLCβ1 also after lithium treatment. The reduced

PLCβ1 mRNA levels after lithium pre-treatment may suggest the involvement of post-transcriptional modification (or delayed translational effects) of PLCβ1 after lithium treatment.

The data from the current study suggest that antidepressant action may include downregulation of PLCβ1 expression and that modulators of the nitric oxide-cGMP pathway (e.g. sildenafil as a PDE5 inhibitor) may exhibit similar properties.

Keywords: *myo*-Inositol, phospholipase C β1, depression, obsessive compulsive disorder, panic disorder, fluoxetine, imipramine, sildenafil, lithium.

Uittreksel

***Titel:* Die modulerende effek van mio-inositol en ander antidepressante op die bRNS vlakke en proteïen uitdrukking van geselekteerde sub-sellulêre ensieme**

mio-Inositol (*m*Ins), 'n natuurlike komponent van die menslike dieët en essensiële voorloper van 'n aantal seinweë, insluitende die van G-proteïen gekoppelde reseptore, het ook getoon dat dit effektief is in die behandeling van psigiatriese toestande soos depressie, obsessiewe kompulsiewe steurnis en panieksteurnis. Moontlik as gevolg van die feit dat *m*Ins 'n eenvoudige isomeer van glukose is, is geen ernstige newe-effekte met die gebruik daarvan gerapporteer nie, selfs met hoë orale dosisse van *m*Ins. Vorige studies het getoon dat die terapeutiese werking van mIns verlaagde serotonien- ($5HT_{2A}$) en muskariene asetielcholienreseptorfunksie insluit. 'n Belangrike seintransduksiesisteem wat moontlik in die werkingsmeganisme van antidepressante betrokke is, is fosfoinositied (PI) omset. In hierdie seinsisteem word PI-fosfolipase C (PLC), wat geïmpliseer is in die werkingsmeganisme van antidepressante en angsiolitika, geaktiveer.

Die werkingsmeganisme van *m*Ins is egter steeds onbekend en benodig verdere ondersoek. In hierdie studie is 'n moontlike modulerende rol van 24-uur voorafbehandelings op 'n menslike neuroblastoom sellyn (SH-SY5Y) met *m*Ins op bRNS vlakke en proteïenuitdrukking van fosfolipase C β1 (PLC β1) en glikogeen sintase kinase 3β (GSK3β) ondersoek. Die effekte van *m*Ins is ook met dié van ander prototipe antidepressante soos fluoksetien ('n selektiewe serotonienopname inhibeerder), imipramien ('n trisikliese antidepressant), litium en nog 'n geneesmiddel met potensiële antidepressant effekte, sildenafil ('n fosfodiësterase 5-tipe inhibeerder) vergelyk. Reële-tyd polimerase kettingreaksie is uitgevoer om die bRNS vlakke te ondersoek, terwyl proteïenuitdrukking in membrane en in die sitosolfraksie van selle met "Western blot" analises gekwantifiseer is.

Die uitdrukking van PLCβ1 is verlaag na voorafbehandeling met imipramien of *m*Ins in kombinasie met fluoksetien. Sildenafil alleen of in kombinasie met *m*Ins het ook die uitdrukking van membraan-gebonde PLCβ1 verlaag. 'n 24-uur voorafbehandeling met litium het nie PLCβ1 uitdrukking betekenisvol verander nie. bRNS vlakke vir die uitdrukking van

PLCβ1 was konsekwent in hierdie bevindinge, behalwe vir die inhibisie van die bRNS vir die uitdrukking van PLCβ1 na litium behandeling. Die verlaagde PLCβ1 bRNS vlakke na litium voorafbehandeling mag moontlik dui op die betrokkenheid van post-transkripsionele effekte van PLCβ1 na litium behandeling.

Die data van die huidige studie toon aan dat antidepressant werking moontlik die afregulering van PLCβ1 uitdrukking insluit en dat moduleerders van die stikstofoksied sintetase cGMP weg (bv. Sildenafil as 'n PDE 5-inhibeerder) moontlik dieselfde eienskappe toon.

Sleutelwoorde: *mio*-inositol, fosfolipase C β1, depressie, obsessiewe kompulsiewe steurnis, paniekversteuring, fluoksetien, imipramien, sildenafil, litium.

Table of Contents

Chapter 1 Introduction

1.1 Problem Statement

Understanding of the neurobiological and genetic underpinnings of major neuropsychiatric disorders has lagged behind the establishment of diagnostic criteria and the development of treatment for these conditions (Rosenberg and Hanna, 2000). It has been estimated that 2-4% of the population suffer from pathological anxiety and frequently no contributing factor can be identified (Leonard, 2003). We also still lack a fundamental understanding of the genes that increase the risk of depression and of the changes in the brain that underlie the diverse symptoms of depression (Nestler *et al.*, 2002).

Mood disorders are among the most prevalent forms of mental illness (Nestler *et al.*, 2002). Over the past ten years it has been widely recognized that depression is one of the most common mental conditions worldwide, having a lifetime prevalence of 17%. It was calculated that depression would soon become the second leading cause of disability (Holsboer, 2001). Panic disorder is a chronic and reoccurring anxiety-related illness (Andersch and Hetta, 2003), while obsessive compulsive disorder (OCD) – another anxiety-related disorder - has a lifetime prevalence of 2-3% (Rosenberg and Hanna, 2000).

Current antidepressant drugs have proven to be effective, but are burdened with slow onset of action and side effects. Above this, it is still unclear by which pharmacological mode of action they exert their clinical effects (Holsboer, 2001).

myo-Inositol (*m*Ins) is the optically inactive stereoisomeric form of inositol and the only form that is nutritionally active (Marcus and Coulston, 1996). *m*Ins is an isomer of glucose and mediates well-established functions in signal transduction and in calcium (Ca^{2+}) homeostasis in the central nervous system (CNS) and non-neuronal tissues (Fisher *et al.*, 2002). It is a simple polyol precursor in the phosphatidyl inositol (PI) cycle, a key second messenger system in the brain (Shimon *et al.*, 1998). Several neurotransmitters cause breakdown of a membrane phospholipid, phosphatidyl inositol biphosphate (PIP_2) into two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG), by activation of the enzyme phospholipase C (PLC) (Belmaker *et al.*, 1995).

A role for *m*Ins, either direct or indirect, has been proposed for a number of disorders in the CNS. Importantly, the administration of high oral doses of *m*Ins has been shown to be effective in the treatment of depression, panic disorder and OCD (Levine, 1997, Belmaker *et al.*, 2002). As a natural sugar, *m*Ins lacks the toxic adverse effects while providing therapeutic benefit in depression, panic disorder and OCD (Parthasarathy *et al.*, 2003). Despite the increasing awareness of *m*Ins as a possible therapeutic intervention in depression, OCD and panic disorder, the mechanism whereby *m*Ins exerts its therapeutic responses remains elusive (Harvey *et al.*, 2002). Recently, Brink *et al.* (2004) have shown that a 24-hour pre-treatment of human neuroblastoma cells with concentrations of 1 and 10 mM *m*Ins (*i.e.* within physiological range) is able to reduce serotonin $5HT_{2A}$ receptor ($5HT_{2A}$-R) and muscarinic acetylcholine receptor (mAChR) function concentration-dependently. Data suggested that *m*Ins reduces $5HT_{2A}$-R function, *inter alia*, by an inhibitory effect at the level of the receptor G_q-protein. Interestingly, the antidepressant fluoxetine, but not imipramine, also inhibits $5HT_{2A}$-R function, whereas both inhibit mAChR function. While *m*Ins appears to be effective only in disorders that are responsive to the selective serotonin reuptake inhibitors, it was also interesting to note that *m*Ins exerts similar effects as fluoxetine on $5HT_{2A}$-R and mAChR function in human neuroblastoma cells (Brink *et al.*, 2004).

1.2 Research objectives

The research objectives of the current study are to investigate the possible modulating effect of 24-hour pre-treatments of human neuroblastoma cells with *m*Ins, in comparison to prototype antidepressants and other selected drugs, on:

- the mRNA levels of phospholipase C-β1 (PLCβ1) and another enzyme implicated in antidepressant action, Glycogen synthase kinase 3β (GSK3β)

- the protein expression of PLCβ1 and GSK3β.

The modulating effect of *m*Ins pre-treatment on the mRNA levels and protein expression will be compared to the modulating effects of the following drugs: fluoxetine, imipramine, sildenafil, lithium and possible potentiating effects of *m*Ins on the potential modulating effects of fluoxetine or sildenafil.

1.3 Project layout

All the experiments were conducted in the Laboratory for Applied Molecular Biology at the Potchefstroom Campus of the North-West University, Potchefstroom, South Africa.

A human neuroblastoma cell line was used and pre-treated with *myo*-inositol (*m*Ins), fluoxetine, imipramine, sildenafil, lithium, fluoxetine + *m*Ins or sildenafil + *m*Ins. and possible potentiating effects of fluoxetine and sildenafil in combination with *m*Ins. Posttranscriptional investigation of the action of the pre-treatments was achieved by using quantitative real-time reverse transcription Polymerase Chain Reaction (real time RT-PCR). Posttranslational investigation of the modulating effects of *m*Ins was performed by using Western Blots to measure the relative expression levels of various proteins in cell membrane and the cytosol fractions of the cells.

Chapter 2 Literature Review

2.1 myo-Inositol

2.1.1 An overview of myo-Inositol

Inositol (hexahydroxycyclohexane) is an isomer of glucose and *myo*-inositol (*m*Ins) is the optically inactive stereoisomeric form and the only form that is nutritionally active (Marcus and Coulston, 1996).

Inositol is a natural constituent of the human diet in amounts of approximately 1 g per day (Freeman *et al.*, 2002), mostly from fruit and plant sources, such as wholegrain cereals and vegetables (Marcus and Coulston, 1996). Approximately 4 g/day inositol could be synthesized *de novo* from glucose by the kidneys if required. Brain and testis also synthesize inositol, but the kidney is by far the major organ involved in its catabolism and excretion (Fisher *et al.*, 2002). There has been no demonstration of a need for dietary supplementation of inositol in human beings, possibly due to its production by human gut bacteria. A high concentration of inositol is also present in breast milk (Marcus and Coulston, 1996).

*m*Ins is present in the phospholipids of cell membranes and plasma lipoproteins in the form of phosphatidylinositol (PI) (Harvey *et al.*, 2002; Marcus and Coulston, 1996). It is the precursor of the phosphoinositide (PI) cycle in the cell and is necessary for the production of two second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Einat and Belmaker, 2001). Since phospholipids are required for the structure of all neuronal membranes, and as they play key roles in the signal transduction processes which form the links between receptor occupancy and neuronal response, abnormalities in the phospholipid metabolism are good candidates for possible biochemical bases of psychiatric disorders (Horrobin and Bennett, 1999).

*m*Ins is also a key metabolic precursor in the PI cycle, a second messenger system for numerous neurotransmitters (Levine, 1997). It also functions as an osmolyte in the nervous system.

It has been demonstrated recently that large doses of inositol possess psychoactive properties (Parthasarathy *et al.*, 2003). As a natural sugar, *m*Ins lacks toxic adverse effects, while providing therapeutic benefit in depression, panic disorder and OCD (Parthasarathy *et al.*, 2003).

2.1.2 The Role of mIns in Anxiety-Related Disorders

A direct or indirect role for *m*Ins has been proposed for a number of disorders of the CNS (Fisher *et al.*, 2002). Decreased cerebrospinal fluid inositol has been reported in depression (Levine, 1997). Shimon *et al.* (1997) found reduced frontal cortex *m*Ins levels in post-mortem brains of suicide victims and bipolar patients.

Belmaker *et al.* (1995) hypothesized that *m*Ins may be deficient in some brain systems in depression. This does not contradict the concept that the antidepressant, lithium, reduces inositol levels, since the PI cycle serves as a second messenger for several balancing and mutually interactive neurotransmitters. Lithium could alleviate depression by reducing inositol in one system without increasing inositol levels above normal levels in another (Belmaker *et al.*, 1995).

Levine (1997) reported a significant overall benefit for inositol compared to placebo in depression, panic disorder and obsessive-compulsive disorder (OCD). Inositol administration, however, is reported to be either ineffective or even contraindicated in conditions such as attention deficit hyperactivity disorder (Levine, 1997), schizophrenia, Alzheimer's disease and autism (Fisher *et al.*, 2002). According to Freeman *et al.* (2002), inositol may play a role in the pathophysiology of anxiety disorders. Interestingly, inositol has long been used as a folk remedy for anxiety and depression in Europe (Belmaker *et al.*, 1995).

The reported beneficial effects of *m*Ins become evident only after 4 to 6 weeks of treatment, a time frame similar to that required for most therapeutic agents (Fisher *et al.*, 2002). Harvey *et al.* (2002) hypothesized two possible mechanisms of action of *m*Ins. The first possibility is that *m*Ins may demonstrate significant inherent pharmacological effects independent of PI turnover. The second possibility is that adaptive changes that follow after chronic exposure suggest that its actions result as a secondary effect after changes in gene expression. Another possible explanation for the therapeutic effect of *m*Ins can be that exogenous inositol regulates phospholipase C (PLC), an idea that is supported by data showing the attenuation of serotonin 5-HT_2 receptor (5-HT_2-R) desensitisation by *m*Ins (Levine, 1997).

A number of different experimental approaches support the idea of behavioural effects of *m*Ins, but its therapeutic mechanism of action in depression, OCD and panic disorder is still unclear.

2.2 Selected Signal Transduction Pathways and Enzymes

2.2.1 Phospholipase C, Isozymes and the Phosphoinositide Metabolic Pathway

Three PLC families have been identified, each constituting more than one isoform (Cocco *et al.*, 1999). The three main families of PI-PLC include the PLC-β, PLC-γ, and PLC-δ isozymes (Horrobin and Bennett, 1999; Pandey *et al.*, 2002), all of which are found in the brain (Horrobin and Bennett, 1999). The gamma isoform is found in neurons throughout the brain, while the beta isoform is found particularly in the cortex and hippocampus and the delta isoform is found particularly in glial cells (Horrobin and Bennett, 1999).

Each isotype of PLC appears to be regulated by different mechanisms (Cocco *et al.*, 1999). PLC-β is activated by receptors that activate α or β subunits of the heterotrimeric G_q family of guanosine triphosphate (GTP) binding proteins (G proteins), while PLC-γ is regulated by receptors and non-receptor tyrosine kinase (Dwivedi *et al.*, 2002). Isozymes of PLC-γ family are activated by phosphorylation of tyrosine residues (Cocco *et al.*, 1999). Little is known about the regulation of PLC-δ, but recent studies suggested that PLC-δ is activated by an atypical G protein, namely G_h, which acts as a transglutaminase (Dwivedi *et al.*, 2002).

All PLC isozymes recognize PIP_2 as a substrate and carry out Ca^{2+} dependent hydrolysis of inositol lipids. These isozymes, however, are differentially regulated and expressed (Dwivedi *et al.*, 2002; Pandey *et al.*, 2002). In the nervous system and in other tissues, PLC plays a central role in signal transduction processes and is linked to glutamate as well as serotonin and muscarinic receptor signalling (Horrobin and Bennett, 1999).

The PI signal transduction system may possibly be involved in the mechanism of action of antidepressants (Gould *et al.*, 2002). In this signalling system, the agonist binds to neurotransmitter receptors, such as 5-HT_{2A}-Rs, 5-HT_{2C}-Rs, α-adrenergic receptors (α-Ars) and muscarinic acetylcholine receptors (mAChRs), resulting in the activation of the receptor-coupled G_q or G_o class of G-proteins (Ackenheil, 2001). This, in turn, activates adenylyl

cyclase (AC) (Lesch, 2001) and the hydrolysing enzyme PI-specific phospholipase C (PI-PLC) (Dwivedi *et al.*, 2002). G-protein-coupled receptors are the starting point of a cascade of intracellular events (Ackenheil, 2001). G-protein signalling is modulated by various regulators of G-protein signalling (RGSs), which are expressed in a region-specific fashion, also in the brain (Lesch, 2001).

There are three major inositol phospholipids (Horrobin and Bennett, 1999). Approximately 5% of cellular PI is phosphorylated at the 4-position (PIP) and another 5% is phospholylated at both the 4 and 5 positions, phosphatidyl-inositol 4,5-biphosphate (PIP_2) (Rameh and Cantley, 1999; Horrobin and Bennett, 1999). Phosphoinositide 3-kinases (PI-3K) catalyzes phosphorylation of the 3'-OH position of *m*Ins lipids that serve as secondary messengers (Djordjevic and Driscoll, 2002). PI plays a crucial role in signal transduction as the precursor of several second-messenger molecules (Fruman *et al.*, 1998).

As discussed in §2.1.1, PI-PLC catalyzes the hydrolysis of PIP_2 into IP_3 and DAG (Gould *et al.*, 2002). DAG is an intermediate in the synthesis of both phospholipids and triglycerides (Horrobin and Bennett, 1999). Both IP_3 and DAG act as second messengers (Pandey *et al.*, 2002, Cocco *et al.*, 2002). After binding with IP_3 receptors, IP_3 mobilizes calcium (Ca^{2+}) from intracellular sources (Mishra and Bhalla, 2002). The action of IP_3 is short-lived, since it is rapidly hydrolyzed to inositiol 1,4-biphosphate (IP_2) and IP and eventually to *m*Ins under influence of the enzyme inositol monophosphatase, which is inhibited by lithium. Alternatively IP_3 is converted to inositol 1,3,4,5-tetrakisphosphate (IP_4) (Brailoiu *et al.*, 2002). IP_4 may in turn be converted to inositol 1,3,4,5,6-pentakisphosphate (IP_5) and inositol 1,2,3,4,5,6-hexakisphosphate (IP_6) (Brailoiu *et al.*, 2003).

The enzyme glycogen synthase kinase 3 (GSK3) is involved in the regulation of at least three intracellular signal transduction cascades. These cascades are responsible for neuronal development, mitogen-activated protein kinase (MAPK) and PI-3K (Ackenheil, 2001).

DAG activates the phosphorylating enzyme, protein kinase C (PKC) (Pandey *et al.*, 2002; Schwartz *et al.*, 2003; Mishra and Bhalla, 2002). PKC activation and Ca^{2+} mobilization are necessary for many cellular functions, for example cell-to-cell communication, secretion and cell growth, differentiation and proliferation (Cocco *et al.*, 1999). PKC in turn activates cAMP response element binding protein CREB (Duman, 2002). DAG is converted to phosphatidic acid (PA) by the enzyme DAG kinase, which is then converted to Cytidine diphosphodiacylglycerol (CDP-DAG), which can interact with free *m*Ins to produce PI. After this process the cycle is being continued (Horrobin and Bennett, 1999).

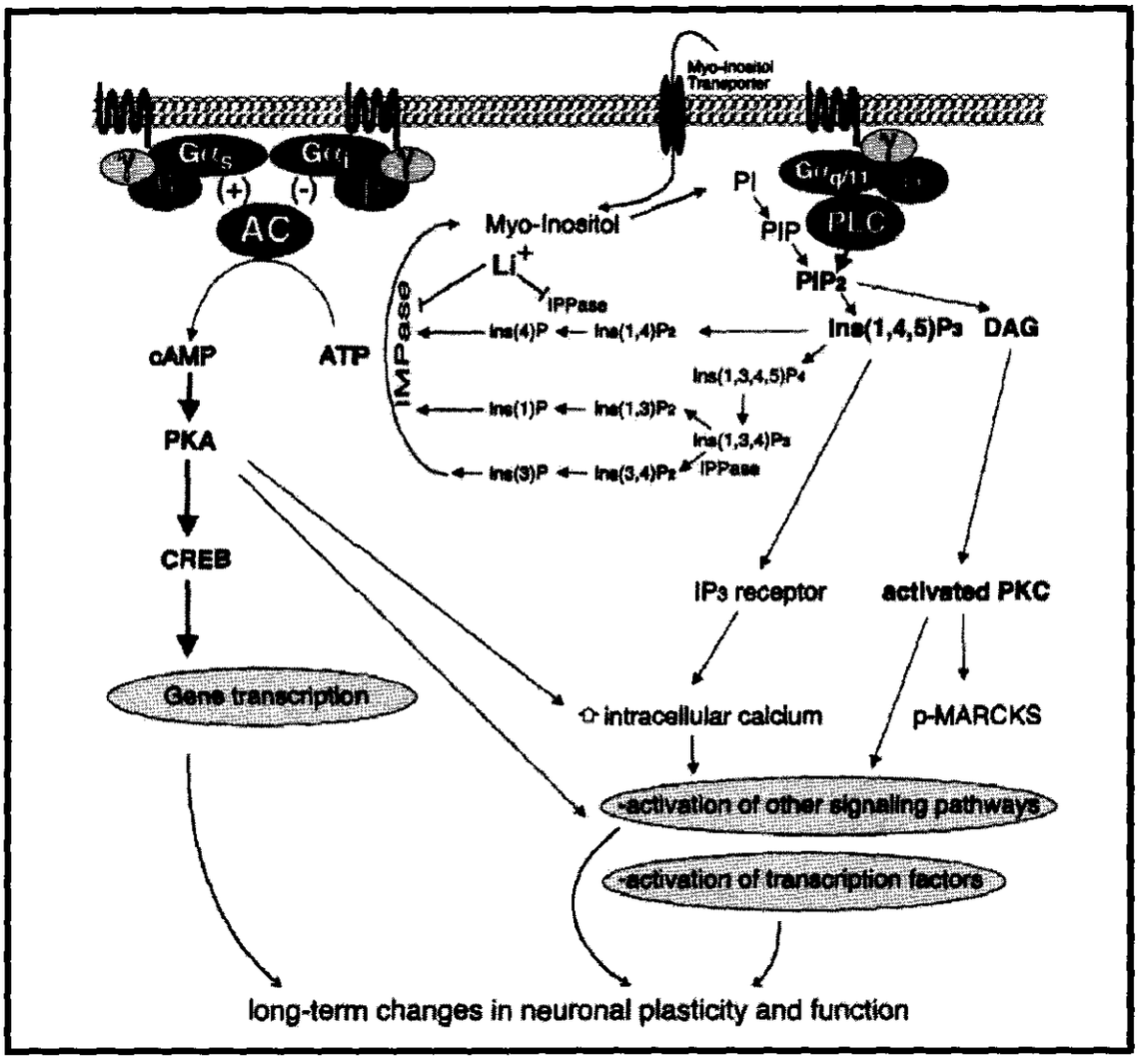


Figure 2-1: *A schematic representation of the PI pathway*

One of the mechanisms by which antidepressants may modulate PI metabolism is through the enzyme PI-PLC. PI-PLC occupies an essential position in the PI signalling system. PI-PLC further has a critical role in mediating various physiological functions, including neurotransmitter release (Brailoiu *et al.*, 2003), cell growth, differentiation, neuronal development and gene expression (Dwivedi *et al.*, 2002).

Although the role of PLC in affective disorders has not been fully investigated, some studies have shown abnormalities of PLC in depression and suicide (Pandey *et al.*, 1999) while others indicate abnormalities of the PI signalling system in patients with unipolar or bipolar disorders (Pandey *et al.*, 2002). A change in PIP_2 levels has been reported in platelets of bipolar patients (Soares *et al.*, 2000). Pandey *et al.* (2002), observed a significantly decrease in PI-PLC activity in membrane and cytosol fractions of platelets from bipolar patients compared with normal control subjects. They further observed that there was no

difference in the protein expression of PLC-β_1 or -γ_1 isozymes in membrane or cytosol fractions, while the protein expression of PLC-δ_1 was decreased in both membrane and cytosol fractions of platelets from bipolar patients. Pandey *et al* (2002) explained the abnormal PI signalling in unipolar patients as a result of alterations in G proteins.

However in a study by Dwivedi *et al.* (2002), chronic antidepressant treatment (desimipramine, fluoxetine, phenelzine, alprazolam and buspirone) decreased PI-PLC activity and specifically decreased the protein level of PLC-β_1 isozyme in membrane and cytosol fractions of the cortex and hippocampus of male rats. Interestingly, all these drugs did not cause any changes in the protein levels of the PLC-γ_1 or PLC -δ_1 isozymes (Dwivedi *et al.*, 2002). Similar changes were found in the mRNA levels of the PLC-β_1 isozyme after chronic treatment with antidepressants, as was observed with its protein levels (Dwivedi *et al.*, 2002). These findings suggest that that antidepressants regulate the transcription of specific PLC-β_1 isozyme and that altered PI-PLC activity could be related to alterations in the expression of this specific PLC isozyme (Dwivedi *et al.*, 2002). As discussed above, PLC-β_1 is primarily activated by G_q proteins, which couple to receptors such as 5-HT_{2A}-Rs, 5-HT_{2C}-Rs, α_1-ARs and mAChRs (Dwivedi *et al.*, 2002). However, taking the different pharmacological profiles of the drugs used in this study by Dwivedi *et al.* (2002), into consideration, it is interesting that drugs from different classes of psychoactive drugs interact with PI-PLC, and specifically at the level of PLC β_1. This study suggests that the decrease in PI metabolism is related to the ability of antidepressants to interact with PI-PLC rather than that this is a receptor-mediated event, which is also supported by the fact that most antidepressants that show a decrease in PI metabolism are not antagonists of 5-HT_{2A} or α-AR

This finding appears to be an indirect effect, since changes in PI-PLC were only observed after chronic treatment (Dwivedi *et al.*, 2002). It is therefore possible that depression is associated with a hyperactive PI signal transduction system and that antidepressants may be alleviating depressive symptoms by decreasing PI-PLC and thus PI hydrolysis (Dwivedi *et al.*, 2002). Dwivedi *et al.* (2002), stated that these changes do not appear to be related to upstream events at the level of receptor upregulation or downregulation and that it could be adaptive or secondary in nature during antidepressant treatment. In conclusion, PLC may be an important target of antidepressant action, which may be relevant to the therapeutic effects of these drugs.

2.2.2 The cAMP-CREB pathway and cell survival

Neurotrophins are a family of regulatory factors that mediate the differentiation and survival of neurons, as well as the modulation of synaptic transmission and synaptic plasticity (Manji *et al.*, 2003). Neurotrophins regulate neuronal development, survival and function in the periphery and CNS (Castrén, 2004). Within the neurotrophic family, brain derived neurotrophic factor (BDNF) is a potent physiological survival factor that has also been implicated in a variety of pathophysiological conditions (Manji *et al.*, 2003).

In the brain, expression and release of BDNF are regulated by neuronal activity, and BDNF has been implicated as a central player in the process of activity-dependent selection of functional neuronal connections during brain maturation (Duman, 2002; Castrén, 2004). The expression of BDNF in the hippocampus, however, is regulated by stress and psychotropic drugs (Duman, 2002).

In contrast to the effects of stress, chronic antidepressant administration increases the expression of BDNF in the hippocampus, as well as frontal cortex (Duman, 2002). BDNF also plays a critical role in learning and memory in the adult brain through similar activity-dependent mechanisms (Castrén, 2004).

The cellular actions of BDNF are mediated through two types of receptors: a high affinity tyrosine receptor kinase (TrkB) and a low affinity pan-neurotrophin receptor (p75). TrkB is preferentially activated by BDNF and neurotrophin 4 or 5 and appears to mediate most of the cellular responses to these neurotrophins (Manji *et al.*, 2003). BDNF and other neurotrophic factors are necessary for the survival and functions of neurons. Acute effects of BDNF include synaptic plasticity, neurotransmitter release and facilitation of the release of glutamate, Gamma-amino butyric acid (GABA) and serotonin (5-HT) (Manji *et al.*, 2003). BDNF's long-term neurotrophic and neuroprotective effects may be important for its putative role in the pathophysiology and treatment of mood disorders (Manji *et al.*, 2003). It is clear that survival-promoting effects are mediated in large by an inhibition of cell death cascades (Riccio *et al.*, 1999).

Increasing evidence suggests that neurotrophic factors inhibit cell death cascades by activating the mitogen-activated protein (MAP) kinase signalling pathway and the PI-3K/Akt pathway. MAP kinase inhibits cell death by increasing the expression of the antiapoptotic protein bcl-2 (Manji *et al.*, 2003). It is clear that the neurotrophic factor/MAP kinase /bcl-2 signalling cascade plays a critical role in cell survival in the central nervous system (CNS) (Yuan *et al.*, 2004) and that there is a fine balance maintained between the levels and

activities of cell-survival and cell-death factors. Modest changes in this signalling cascade or in the levels of the bcl-2 family of proteins may affect cellular viability (Manji *et al.*, 2003). Therefore, neurotrophic signalling molecules may play important roles in the treatment of mood disorders (Manji *et al.*, 2003).

Antidepressant treatment increases synaptic levels of norepinephrine (NE) and 5-HT via blocking the reuptake or breakdown of these monoamines (Castrén, 2004). This results in activation of intracellular signal transduction cascades, one of which is cyclic adenosine monophosphate (cAMP)-CREB cascade. Chronic antidepressant treatment increases G_s coupling to AC, particulate levels of cAMP-dependant protein kinase (PKA), and CREB (Manji *et al.*, 2003). In addition to CREB's regulation by the cAMP cascade, other signal transduction pathways like Ca^{2+}-calmodulin-dependent kinase, PKC, ribosomal S6 kinase as well as cAMP-dependent kinase also activates CREB (Duman, 2002). Ca^{2+}-dependent protein kinases can be activated by the PI pathway or by glutamate inotropic receptors (NMDA) (Manji *et al.*, 2003). CREB is considered the prime candidate in mediating the antidepressant-induced increase in BDNF mRNA (Castrén, 2004). However, not only does CREB activate BDNF production, but BDNF also induces CREB phosphorylation (Castrén, 2004). Therefore, these two key molecules form a positive feedback loop that may be critical in the trophic effects of antidepressants (Castrén, 2004).

The first step of 5-HT biosynthesis in serotonergic neurons is catalysed by the rate-limiting enzyme tryptophan hydroxylase (TPH). Abnormalities in TPH have been implicated in a wide range of psychiatric disorders (Lesch, 2001).

Treatment of depression is attained by providing both trophic and neurochemical support. The trophic support restores normal synaptic connectivity, thereby allowing the chemical signal to reinstate the optimal functioning of critical circuits necessary for normal affective functioning (Manji *et al.*, 2003). BDNF also facilitates the release of neurotransmitters that act on this restored, intact circuit. Acute reduction in synaptic 5-HT levels via its effects on reducing BDNF levels is capable of rapidly reducing the release of a number of neurotransmitters (Manji *et al.*, 2003). The fact that trophic changes probably take time to develop and mature might at least partially explain the delay in the development of the clinical antidepressant effect (Castrén, 2004).

Thome *et al.* (2000) demonstrated that antidepressant treatment *in vivo* increases CREB phospholylation and CRE-mediated gene expression in mouse limbic brain regions. Upregulation of BDNF is dependent on chronic treatment, consistent with the therapeutic action of antidepressants (Manji *et al.*2003). Duman (2002) has found that the function and

expression of CREB is upregulated by chronic antidepressant treatment, including *l*-norepinephrine (*l*-NE) reuptake inhibitors and serotonin selective reuptake inhibitors (SSRIs). In addition, CREB has been shown to modulate antidepressant drug activity (Benedetti *et al.*, 2004). Duman's (2002) hypothesis is that downregulation of CREB could contribute to the pathophysiology of depression and that upregulation of this transcription factor could contribute to the therapeutic response. Furthermore, CREB mRNA levels and phosphorylation are reduced in post-mortem brain samples of depressed patients (Castrén, 2004). CREB mRNA is induced by antidepressants, and expression of CREB in the hippocampus produces a similar kind of behavioural response to that of antidepressants (Castrén, 2004). Recent studies demonstrate that the cAMP-CREB cascade, a pathway involved in cell survival and plasticity, is upregulated by antidepressant treatment (Duman *et al.*, 1999). Studies showed that upregulation of the cAMP-CREB cascade and BDNF increases performance on behavioural models of depression and this supports the role of these pathways in the action of antidepressant treatment (Duman *et al.*, 1999). It has also been observed that induced CREB overexpression in the dentate gyrus results in an antidepressant-like effect in the learned helplessness paradigm and the forced swim test in rats (Manji *et al.*, 2003). Chen *et al.* (2001) reported increased hippocampal BDNF expression in post-mortem brain of subjects treated with antidepressants at the time of death versus antidepressant-untreated subjects. Chronic administration of different classes of antidepressant treatment, including NE, SSRIs and electroconvulsive seizures, increases the proliferation and survival of new neurons (Manji *et al.*, 2003). Mood disorders and their experimental models are characterized by reduced neuronal activity and synaptic connectivity (Castrén, 2004). Antidepressants, through their BDNF- and CREB-mediated trophic effects, could boost synaptogenesis and help to restore the neuronal connections lost in depression through inadequate neuronal activity (Castrén, 2004).

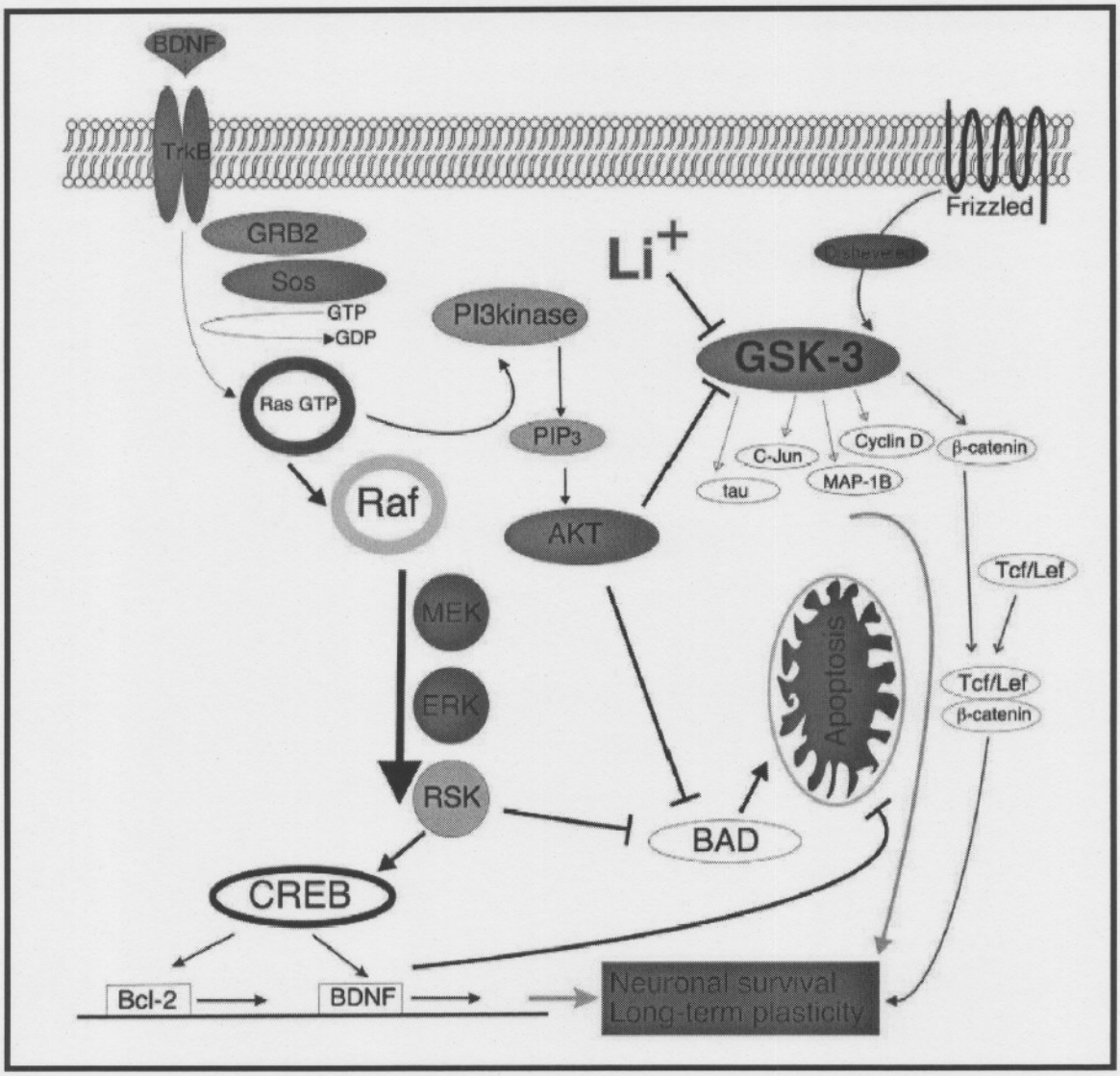


Figure 2-2: *A schematic representation of the cAMP-CREB pathway (Gould et al., 2002).*

2.2.3 Glycogen synthase kinase and the Wnt signalling pathway

The Wingless signalling pathway (Wnt) cascade is a central signal transduction pathway mediating brain development by involvement in cell proliferation, cell adhesion and synapse rearrangement (Nadri *et al.*, 2004). The Wnt pathway is operative in adult organisms and in the adult nervous system, while the Wnt signalling pathway also plays a major role in cell fate determination during early embryonic development (Gould *et al.*, 2002). GSK3 is a component of the Wnt signalling cascade (Nadri *et al.*, 2004), where the Wnt pathway is responsible in for suppressing of GSK3β (Harvey *et al.*, 2002). .

Secreted Wnt glycoproteins interact with the frizzled family of receptors to deactivate GSK3, thus preventing the phosphorylation and subsequent degradation of β-catenin by this constitutively active enzyme (Gould *et al.*, 2002). GSK3 is found in two nearly identical isoforms in mammals, α and β (Benedetti *et al.*, 2004, Barry *et al.*, 2003) and GSK3β is highly expressed in brain tissue (Ryder *et al.*, 2004; Nadri *et al.*, 2004). GSK3 is rather unusual among kinases in that it is constitutively active (Gould *et al.*, 2002). Thus, most intracellular signals to GSK3 inactivate the enzyme (Gould *et al.*, 2002). GSK3 is suggested to play a role in multiple cellular processes including metabolism, proliferation, differentiation and development (Agam *et al.*, 2003). In all these processes GSK3 takes part in signal transduction cascades (Agam *et al.*, 2003). GSK3 also plays a role in mediating signals from insulin/PI3K that effect glycogen synthesis (Gould *et al.*, 2002). Signals deactivating GSK3 arise from numerous growth factors and developmental signals (Gould *et al.*, 2002).

A number of endogenous growth factors (like BDNF) utilize the PI3K signalling cascade as a major effector system. Thus, growth factors may bring about many of their neurotrophic/neuroprotective effects, at least in part, by GSK3 inhibition (Gould *et al.*, 2002). BDNF, which is modulated by antidepressants and produces antidepressive-like activity in preclinical behavioural models, is able to inhibit GSK3 β (Benedetti *et al.*, 2004).

GSK3 phosphorylates – and thereby inactivates – many other targets including transcription factors and cytoskeletal proteins (Jope, 2004). A rapidly increasing amount of evidence suggests that GSK3 plays important roles in regulating neuronal survival and synaptic plasticity (Benedetti *et al.*, 2004). Accumulating evidence suggests that lithium may have some neuroprotective effects, possibly due to inhibition of GSK3.

*m*Ins, through a PI-independent mechanism, prevents the downstream effects of GSK3β on the Wnt pathway. Harvey et al. (2002) stated that exogenous *m*Ins could cause an indirect modulatory action on GSK3β. This effect on GSK3β, and the subsequent effects of the latter on transcription factor activation may represent an important molecular site of action for *m*Ins (Harvey et al., 2002).

2.2.4 The role of Serotonin Receptors

All 5-HT receptor subtypes, except for 5-HT_3 receptor, are coupled to signal-transducing heterotrimeric G-proteins (Lesch, 2001), while 5-HT_3 receptors are 5-HT-gated ion channels (Raymond et al., 2001). Recent studies have revealed a rich diversity of coupling mechanisms for each 5-HT receptor subtype (Raymond et al., 2001).

There are five members of the 5-HT_1 receptor family (Raymond et al., 2001). The 5-HT_1 subtypes (1A, 1B, 1D, 1E) couple primarily through $G_{i/o}$-proteins to the inhibition of AC (Barnes and Sharp, 1999). cAMP modulates the expression of genes containing a CRE in their 5'-flanking regulatory regions (Lesch, 2001). The 5-HT_{1A} receptor has been reported to activate or inhibit various enzymes, channels, and kinases, and to stimulate or inhibit production of diverse soluble second messengers. This receptor has been reported to inhibit PLC also and to stimulate nitric oxide synthase (NOS). It can activate PKC and MAP kinase. The 5-HT_{1A} receptor can inhibit or stimulate Ca^{2+} mobilization and activate or inhibit PI hydrolysis (Raymond *et al.*, 2001).

All three 5-HT_2 receptor subtypes couple positively to PLC, leading to increased accumulation of inositol phosphates and intracellular Ca^{2+} (Barnes and Sharp, 1999). 5-HT_{2A-C} receptors stimulate PLC through G_q protein isotypes with subsequent activation of protein kinase C (PKC) (Lesch, 2001). 5-HT modulates presynaptic and postsynaptic gene expression in the brain. 5-HT_{2A-C} receptors induce via PLC-activated second messengers, mitogenesis in non-neuronal cells and are involved in the neural plasticity of post-mitotic neurons. The pattern of gene expression is different from chronic against acute stimulation of 5-HT receptor subtypes (Lesch, 2001). Complex transcriptional control mechanisms have been shown to be responsible for cell-selective and antidepressant-induced regulation of the 5-HT_{2A} receptor. Downregulation of the 5-HT_{2A} receptor by antagonists, such as the atypical antidepressant mianserin, is mediated by a drug response sequence in the transcriptional apparatus of the receptor gene (Lesch, 2001). Of current interest is evidence that stimulation of the 5-HT_{2A} receptor causes activation of a biochemical cascade leading to altered expression of a number of genes, including that of BDNF (Barnes and Sharp). These changes may be linked, at least in part, to the increase in the expression of BDNF seen following repeated treatment with antidepressants (Barnes and Sharp). Serotonin plays a crucial role in the aetiology of depression (Leonard, 2003).

Multiple mechanisms contribute to homologous desensitisation of 5-HT_2 receptors including activation of protein kinase C (PKC) (Rahman and Neuman, 1993).

An abnormal 5-HT receptor function is indicated by an increase in the density of cortical 5-HT_{2A} receptors in the brains of suicide victims and also on the platelet membrane of depressed patients (Leonard, 2003). The function of the 5-HT_{2A} receptor also appears to be subnormal in the untreated patient as shown by reduced aggregatory response to the addition of 5-HT *in vitro*, but normalizes when the patient recovers. As the number of 5-HT_{2A} receptors on the platelet membrane of depressed patients is increased, this finding suggests

that the G-protein transducer mechanism, which links the receptor to the second messenger PI system within the platelet, is possibly defective in depression (Leonard, 2003). These studies of platelet function before, during and following treatment can give important information of the biochemical processes that may be causally related to depression. However, it is still uncertain how the changes in platelet function precisely reflect those occurring in the brain (Leonard, 2003).

Thus when the results of the studies on platelets, lymphocytes, changes in cerebrospinal fluid metabolites of brain monoamines and the post-mortem studies are taken into account it may be concluded that a major abnormality in both noradrenergic and serotonergic function occurs in depression, and that such changes could be causally related to the disease process (Leonard, 2003).

Brink *et al.* (2004) found that *m*Ins and fluoxetine reduces 5-HT_{2A} receptor (5-HT_{2A}-R) function, while imipramine increased 5-HT_{2A}-R function.

2.2.5 The role of muscarinic cholinergic receptors (mAChR)

Anticholinergic activity may contribute to the efficacy of antidepressant drugs (Daws and Overstreet, 1999). Muscarinic receptors also stimulate PLC mediated PI hydrolysis (Rahman and Neuman, 1993). Brink *et al.* (2004) found that *m*Ins inhibits mAChR function. . Brink *et al.* (2004) found that fluoxetine and imipramine (at high concentrations), reduce muscarinic acetylcholine receptor (mAChR) function.

2.3 Anxiety and anxiety-related disorders

Depression, panic disorder and OCD are either specific anxiety disorders (panic and OCD) or disorders that present with significant comorbid anxiety symptoms (depression) (Harvey *et al.*, 2002)

Several neurotransmitter systems and their metabolic pathways have been elucidated in mood disorders, including glutamate, GABA, serotonin (5-HT), *l*-NE and dopamine, as have the membrane-bound signal transduction elements and the intracellular signalling systems, which modulate gene transcription and protein synthesis (Tamminga *et al.*, 2002).

Virtually every physical and psychiatric disorder has a genetic component (Gerson, 1995). However, the vast majority of these diseases have a complex pattern of inheritance and there is no evidence that a single genetic locus is responsible for any of the major psychiatric

disorders (Horrobin and Bennett, 1999; Ackenheil, 2001). Rather, it appears that multiple alleles occurring at multiple sites within the genome interact to produce a vulnerability to the disorder (Manji *et al.*, 2003; Leonard, 2003).

2.3.1 Depression

2.3.1.1 Prevalence of depression

Major depression is one of the most common mental illnesses (Baldessarini, 1996) and one of the most pervasive and costly brain diseases (Tamminga *et al.*, 2002). The illness has a 10% mortality due to suicide and a presumption of increased rates of serious accidents among persons with active mood disorders (Tamminga *et al.*, 2002).

Depression and bipolar disorder (also well known as manic-depressive illness) are highly heritable disorders, with genetic factors comprising roughly 50% of the risk for depression and as much as 80% of the risk for bipolar disorder (Nestler *et al.*, 2002). Family studies, adoption studies and twin studies leave little doubt that major depression has substantial genetic components (Horrobin and Bennett, 1999; Ackenheil, 2001).

2.3.1.2 Symptoms

Major depression is characterized by feelings of intense sadness and despair, mental slowing and loss of concentration, pessimistic worry, agitation, and self-deprecation (Baldessarini, 1996). Physical changes also occur, particular in severe or "melancholic" depression; these include insomnia or hypersomnia, anorexia and weight loss (or sometimes overeating), decreased energy and libido, and disruption of the normal circadian rhythms of activity, body temperature and many endocrine functions (Baldessarini, 1996). As many as 10% to 15% of individuals with this disorder display suicidal behaviour during their lifetime

2.3.1.3 Aetiology of depression

The role of the cholinergic system in depression

An enhanced activation of the anterior pituitary gland, as suggested by increased growth hormone secretion, is caused after administration of the short acting reversible cholinesterase inhibitor pyridostigmine (Leonard, 2003). For this reason, Leonard (2003) supports the cholinergic hypothesis of depression. This suggests that the muscarinic

receptors are supersensitive in the depressed patient. However, the mechanisms whereby the receptors are normalized by chronic (but not acute) antidepressant treatment differ and in most cases are unlikely to be due to direct anticholinergic action. It has been assumed that depression occurs as a result of an imbalance between the central noradrenergic and cholinergic systems (Dazzi *et al.*, 2001). In depression the activity of the noradrenergic system is decreased and in mania it is increased (Harvey, 1997). As most antidepressants have been shown to enhance noradrenergic function, it is hypothesized that the functional reduction in cholinergic activity arises as a consequence of the increase in central noradrenergic activity (Leonard, 2003).

The role of the monoamines in depression

The aetiology of depressive illness has been linked with the brain monoaminergic neuronal dysfunction (Avissar and Schreiber, 2002). Agents that act by various mechanisms to increase synaptic concentrations of monoamines can improve the symptoms of depression. This observation led to the adoption of the monoamine hypothesis of depression (Castrén, 2004). This hypothesis predicts that the underlying pathophysiological basis of depression is depletion in the levels of 5-HT, *l*-NE and/or dopamine in the central nervous system and that antidepressants would restore normal function (Avissar and Schreiber, 2002, Stahl, 2002).

The role of NE in depression and stress is linked to the neuroanatomical structure of the central NE system. The effects of chronic stress on depression models led to differential changes in NE function which can be attenuated by anxiolytics or antidepressants (Brunello *et al.*, 2002). The finding that the density of beta adrenoceptors is increased in cortical regions of the brains from suicide victims who had suffered from depression (Leonard, 2003) is evidence of disturbed noradrenergic function, which is associated with some of the symptoms of the illness (Ackenheil, 2001). These observations are supported by the increase in the density of beta adrenoceptors on the lymphocytes of untreated depressed patients. As the density of these receptors is normalized by effective antidepressant treatment, it has been postulated that changes in the beta receptor density may be a state marker of the condition (Leonard, 2003). Chronic treatment with antidepressant drugs and ECT decreases the activity of NE-dependant AC and downregulates the β1-adrenergic receptors in rat forebrain (Brunello *et al.*, 2002).

The central noradrenergic function is decreased in depression (Ackenheil, 2001), an event leading to the increase in the density of the postsynaptic beta adrenoceptors that show

adaptive changes in response to the diminished synaptic concentration of the transmitter (Leonard, 2003).

According to Achenheil (2001) lower α_2-receptor sensitivity is observed in depressive states and a higher noradrenaline activity in manic states of bipolar depression.

There is also evidence that the density of muscarinic receptors is increased in limbic regions of depressed patients who have committed suicide. If it is assumed that such a change reflects an increased activity if the cholinergic system, it could help to explain the reduced noradrenergic function as there is both clinical and experimental evidence to suggest that increased central cholinergic activity can precipitate depression and reduce noradrenergic activity (Leonard, 2003).

Serotonin and its role in depression

Serotonin is believed to play a multifunctional role in depression (Ackenheil, 2001, Harvey *et al.*, 2002), which is to be anticipated from its involvement in the physiological process of sleep, mood, vigilance, feeding and possibly sexual behaviour and learning, all of which are deranged to varying extents in severe depression (Leonard, 2003). However, the involvement of precise serotonin receptor subtypes in depression, and in the action of antidepressants, is still far from clear (Leonard, 2003; Ackenheil, 2001).

Several types of antidepressant treatments enhance 5HT neurotransmission in the rat hippocampus. This net effect that is common to the major types of antidepressant treatments is, however, mediated via different mechanisms (Blier and De Montigny, 1999). While there is evidence that most antidepressants show only a low affinity for the 5-HT_1 sites and there is experimental evidence to show that chronic antidepressant treatment results in hyposensitivity of presynaptic 5-HT_{1A} receptors (Leonard, 2003), and that TCAs (independent of their capacity to inhibit the reuptake of 5HT and/or NE) progressively enhance the responsiveness of postsynaptic 5-HT_{1A} receptors in the hippocampus (Blier and De Montigny, 1999). This sensitisation to 5HT, after TCA treatment, occurs with a time course (2-3 weeks) that is congruent with the delayed onset of action of these drugs in major depression (Blier and De Montigny, 1999). Desensitisation of the $5HT_{1A}$ autoreceptors occurs after administration of antidepressant drugs (Blier and De Montigny, 1999).

Studies have implicated central 5-HT_{2C} receptors in the control of anxiety, depression and schizophrenia (Wood *et al.*, 2001). The number of 5-HT_2 receptors increases in response to chronic antidepressant and lithium treatment, although chronic electroconvulsive shock

results in a decrease in the receptor number (Leonard, 2003). The density of 5-HT_2 receptors on the platelet membrane in untreated depressed and panic patients is increased (Leonard, 2003). However, the number of receptors normalizes on effective, but not ineffective, treatment. The increase in the 5-HT_2 receptor number, and decrease in their responsiveness to serotonin, in the untreated depressed patient may suggest an abnormality in the coupling mechanism between the receptor site and the PI second messenger system that brings about the platelet shape change underlying aggregation (Leonard, 2003).

It has been hypothesized that depression could arise from a pathological enhancement of 5-HT_2 receptor function. This view would agree with the observations that the functional activity of 5-HT_2 receptors on the platelet membrane is enhanced in depression and their density is increased in the frontal cortex of brains of suicide victims (Leonard, 2003).

The link between the serotonergic and noradrenergic systems

It has been hypothesized that the chronic administration of SSRI antidepressants, such as fluoxetine, slowly desensitize the inhibitory 5-HT_{1B} receptors and thereby enhance serotonin release (Leonard, 2003). There is experimental and clinical proof that the 5-HT_{1A} receptors play an essential role in both anxiety and depression (Leonard, 2003). The 5-HT_{1A} somatodendritic receptors inhibit the release of serotonin and it is assumed that the enhanced release of the transmitter following the chronic administration of the SSRIs is a consequence of the adaptive down-regulation of the inhibitory 5-HT_{1A} receptors (Leonard, 2003).

The validity of this hypothesis is supported by the pharmacological effect of 5-HT_{1A} antagonists (Leonard, 2003). The therapeutic efficacy of SSRIs is enhanced and in some studies the time of onset of the peak therapeutic effect is reduced with the beta-adrenoceptor antagonist and 5-HT_{1A} antagonist, pindolol, in combination with FLX or paroxetine (Leonard, 2003). According to Leonard (2003), some studies have provided evidence that 5-HT can also regulate dopamine turnover and such findings imply that the effects of some antidepressants, that show a clear selectivity for the serotonergic system, could be equally ascribed to a change in dopaminergic function in mesolimbic and mesocortical regions of the brain.

2.3.1.4 Treatment of Depression

Patients often respond well to antidepressant drugs or, in severe cases, to ECT (Baldessarini, 1996). The following agents are used in the treatment of depression.

Tricyclic antidepressants (TCAs)

The first agents used successfully were the TCAs and these have been widely used for the treatment of major depression (Baldessarini, 1996). They can be divided into two main classes. The tertiary amines (imipramine) are drugs that are generally dual (i.e., 5-HT and *l*-NE) reuptake inhibitors, are metabolized to secondary amines and have a high burden of anticholinergic side effects. The secondary amines are generally more selective at blocking *l*-NE reuptake with somewhat reduced anticholinergic side effects (Tamminga *et al.*, 2002).

The therapeutic efficacy of the TCAs has been ascribed to their ability to inhibit the reuptake of noradrenaline and serotonin into the neuron following the release of these transmitters into the synaptic cleft (Leonard, 2003). The excellent clinical efficacy of the TCAs has been well documented and their pharmacokinetic profiles are favourable (Manji *et al.*, 2003). These drugs inhibit muscarinic receptors, histamine type-1 receptors and also display alpha-1 adrenoceptor antagonism (Leonard, 2003). The TCAs are less desirable however due to significant cholinolytic, alpha adrenolytic and cardiac suppressant actions, a narrow therapeutic index, high side effect profile, a clumsy dosing schedule with dosage instability and a high incidence of drug interactions (Harvey, 1997).

The glutamatergic system also plays a role in the action of antidepressants. Evidence shows that TCAs inhibit the binding of the selective NMDA ligand, dizolcipine, to the ion channel of the main glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor in the brain (Leonard, 2003). Such that antidepressants may act as functional NMDA receptor antagonists Leonard (2003).

Monoamine Oxidase Inhibitors (MAOI)

Inhibitors of monoamine oxidase, which increase the brain concentration of many amines, have also been used in depression (Baldessarini, 1996). Irreversible MAOIs inhibit the enzymatic degradation of both monoamine oxidase A (MAOA) and monoamine oxidase B (MAOB) (Tamminga *et al.*, 2002). MAOA oxidizes 5-HT, *l*-NE as well as dopamine (Lesch, 2001). Abnormalities in MAOA activity have been implicated in a wide range of behavioural traits and psychiatric disorders (Lesch, 2001).

There is evidence that MAOIs are more effective than TCAs (and perhaps SSRIs) for atypical depression (characterized by hypersomnia, hyperphagia, reverse diurnal mood variation, and prominent fatigue), as well as in persons who do not respond to other antidepressants (Tamminga *et al.*, 2002).

Selective Serotonin Reuptake Inhibitors (SSRIs)

Recently, after decades of limited progress, a series of innovative antidepressants, the SSRIs, emerged (Nestler *et al.*, 2002). The SSRIs are clearly the drug treatment of choice for all forms of depression (Tamminga *et al.*, 2002). They have a much more benign side effect profile than TCAs and, largely for this reason, has replaced TCAs as first line therapy (Tamminga *et al.*, 2002).

There is no direct relationship between the potency of the drug to inhibit 5-HT reuptake *in vitro* and the dose necessary to relieve depression in the clinic (Leonard, 2003). In experimental studies, it is clear that the increased release of 5-HT from the frontal cortex only occurs following the chronic administration of any of the SSRIs (Leonard, 2003). Thus the inhibition of 5-HT reuptake may be a necessary condition for the antidepressant activity, but it is not sufficient in itself (Leonard, 2003).

The 5-HT transporter removes 5-HT from the synaptic cleft and determines the magnitude and duration of postsynaptic receptor-mediated signalling, thus playing a pivotal role in the fine-tuning of 5-HT neurotransmission. The 5-HT transporter is also the initial target for several antidepressant drugs (like fluoxetine) (Lesch, 2001). Besides their direct inhibitory action on the serotonin transporter (Lesch, 2001), they also have an effect on other neurotransmitter systems, which may have some clinical significance (Leonard, 2003).

In addition to their proven efficiency in the treatment of all types of depression, the SSRIs have been shown to be the drug of choice in the treatment of panic disorder, OCD, bulimia nervosa and post-traumatic stress disorder (Leonard, 2003).

Lithium

Lithium is a mood-stabilizing medication commonly used in patients suffering from manic-depressive disorder and other psychiatric disorders (Parthasarathy et al., 2003) and it is the first drug of choice for long-term treatment of bipolar disorders (Ackenheil, 2001). Lithium is the most effective treatment for reducing both the frequency and severity of recurrent affective episodes (Manji *et al.*, 1996).

A large amount of research implicates possible effects of Lithium on G-protein mediated signalling (Gould *et al.*, 2002). Lithium decreases the binding of the G-protein α-subunit to GTP (Ackenheil, 2001), but does not change the density of G-protein coupled receptors after chronic therapy (Gould *et al.*, 2002). Many transcriptional and posttranscriptional events

regulate the expression and function of G-proteins, resulting in a virtually unlimited number of possibilities whereby G-protein activity can be modulated in cells (Manji *et al.*, 1996). Thus, the changes observed in G-protein coupled signalling may be due to indirect effects from other biological targets, for example enzymes (Gould *et al.*, 2002, Manji *et al.*, 1996).

Lithium inhibits inositol polyphosphate 1-phosphate (IPPase) and inositol monophosphate phosphatase (IMPase) (Agam *et al.*, 2003). Lithium's direct effect on IMPase and secondarily IPPase led to the inositol depletion hypothesis of lithium's action (Gould *et al.*, 2002, Manji *et al.*, 1996). IMPase is the final inositolpolyphosphate phosphatase prior to conversion to inositol (Freeman *et al.*, 2002), while IPPase removes a phosphate from IP_2 (Gould *et al.*, 2002). As previously discussed both these steps are critical in the maintenance of the PI cascade (Gould *et al.*, 2002).

IMPase is a pivotal enzyme in the brain inositol signalling system and appears to be the key enzyme required for the replenishment of brain inositol implicated in neuronal signalling (Parthasarathy *et al.*, 2003). Although there are at least two IMPases (IMPase 1 and 2), IMPase 1 is the predominant lithium-sensitive enzyme in the brain (Parthasarathy *et al.*, 2003). IMPase 1 is involved in the regulation of downstream processes of inositol-based second messenger system and is primarily responsible for releasing free *m*Ins from several of its inositol monophosphates after brain receptor stimulation, or from glucose through the de novo pathway (Parthasarathy *et al.*, 2003). 3' (2')-Phosphoadenosine 5'-phosphate (PAP) is a recently described lithium-inhibitable enzyme. Lithium inhibition of PAP phosphatase may lead to altered gene expression, altered sulphation processes, or altered PI second messenger function (Agam *et al.*, 2003). Agam *et al.* (2003) found that PAP phosphatase protein levels, but not its enzymatic activity, were significantly reduced in bipolar patients and they found no correlation between PAP phosphatase protein levels and PAP phosphatase enzyme activity. Inhibition of PAP phosphatase by lithium may also affect the PI cycle by a direct or indirect path (Agam *et al.*, 2003).

This inositol depletion hypothesis of lithium's action suggests that lithium, via inhibition of IMPase, decreases the availability of *m*Ins, and thus the amount of PIP_2 available for G-protein mediated signalling events that rely upon this pathway (Ackenheil, 2001). It is hypothesized that the brain would be especially sensitive to lithium, due to *m*Ins's relatively poor penetration across the blood brain barrier (Gould *et al.*, 2002, Belmaker *et al.*, 1998). In support of this hypothesis, lithium has consistently been shown to decrease free inositol levels in brain sections, and in the brains of rodents treated chronically with lithium (Manji *et al.*, 1996, Belmaker *et al.*, 1998).

Belmaker *et al.* (1998) found a reduction in bipolar affective disorder in suicide victims in the frontal cortical inositol levels as compared with controls, with a similar but statistically nonsignificant trend in occipital cortex. Lithium decreased *m*Ins in human subjects after five days of treatment, suggesting that any decrease in PIP_2 signalling is upstream of the downstream therapeutically relevant targets (Moore *et al.*, 1999). While lithium does appear to lower inositol levels, there is little evidence that PIP_2 mediated signalling is altered by lithium (Gould *et al.*, 2002). It has been argued, however, that a lithium-induced depletion of inositol might be limited to selected brain areas or even cells that might be particularly vulnerable to this effect because of restricted inositol supply and/or increased activity of the inositol phospholipid second messenger system (Belmaker *et al.*, 1998). Several intracellular pools of inositol may exist and the reduction on frontal cortex may be specific to a pool critical for neuronal second messenger function (Belmaker *et al.*, 1998). Regardless of negative findings, inositol depletion remains a viable hypothesis for the mechanism of action of lithium (Manji *et al.*, 1996).

After chronic treatment, a lithium-induced decrease in inositol is significantly evident in the hypothalamus (Belmaker *et al.*, 1998). However although chronic lithium administration may affect receptor mediated PI signalling, these effects are unlikely to be simply due to inositol depletion in the CNS. Parthasarathy *et al.* (2003) found that IMPase activity increased substantially in various brain regions after chronic lithium treatment. They concluded that this finding was due to an adaptational response to chronic lithium treatment and may involve direct or indirect stimulation of the gene or turnover of the enzyme.

Elevated intracellular free Ca^{2+} concentrations in platelets and lymphocytes of manic and depressed bipolar patients were reported (Ackenheil, 2001). IP_3 binds to a receptor that liberates Ca^{2+} from its intracellular storage places and that explains why the increased PI system activity is accompanied by elevated intracellular Ca^{2+} levels (Ackenheil, 2001).

Additionally, in some tissues, $G_{\alpha s}$ protein couples to Ca^{2+} channels and in this way, increases intracellular Ca^{2+} levels. Both increased PI signalling as well as increased levels of G_{α} subunits in bipolar patients lead to higher cytosolic Ca^{2+} levels (Ackenheil, 2001).

Lithium also inhibits GSK3β, (Meijer *et al.*, 2004) a crucial kinase that functions as an intermediary in numerous intracellular signalling pathways (Gould *et al.*, 2002). The lithium effect on GSK3 can explain the teratogenic effects of lithium as well as the therapeutic effect (Ackenheil, 2001). The teratogenic effects may result from the activation of the Wnt pathway, which is responsible for development, whereas the therapeutic effects are mediated via the MAPK and the PI3 signal transduction systems (Ackenheil, 2001).

Myo-Inositol

As discussed previously, *m*Ins has been implicated in the treatment of depression (Levine, 1997).

The long-term mechanism of action of antidepressants

One of the most important areas of molecular genetics concerns the role of regulatory sequences that surround the sections of the gene that encode the amino acid sequence of a protein. These regulatory sequences are activated or inactivated by specific transcription factors and it is the complex interaction of regulatory sequences and transcription factors that underlie the adaptation of brain function to the effects of some psychotropic drugs. For example it is well known that the optimal response to an antidepressant or neuroleptic drug requires several weeks of treatment. Such adaptive changes are probably a reflection of the molecular genetics of neurotransmitter function and may help to explain the lack of success in developing antidepressants or neuroleptics that have a rapid therapeutic action (Leonard, 2003).

Most antidepressants exert their initial effects by increasing the intrasynaptic levels of 5-HT and/or NE (Yuan *et al.*, 2004). Their clinical antidepressant effects are only observed after chronic administration (Manji *et al.*, 2003). Antidepressant therapy is usually associated with a slow onset of action over 2 to 3 weeks before the optimal beneficial effect is achieved (Dwivedi *et al.*, 2002). The delay in the onset of the therapeutic response cannot be easily explained by the pharmacokinetic profile of the drugs as peak plasma concentrations are usually reached in 7 to 10 days (Leonard, 2003). In addition, the 2-3 week delay is also seen in many, though not all, patients given electroconvulsive therapy (Leonard, 2003).

In an attempt to explain the reason for the delay in the onset of the therapeutic effect of antidepressants, which is undoubtedly unrelated to the acute actions of these drugs on monoamine reuptake transporters or intracellular metabolising enzymes, emphasis has moved away from the presynaptic mechanism leading to the release of the monoamine transmitters to the adaptive changes that occur in presynaptic and postsynaptic receptors that govern the physiological expression of neurotransmitter function (Leonard, 2003). This delayed therapeutic action could result from either the indirect regulation of the other neuronal signal transduction systems (Ackenheil, 2001) or the regulation of gene transcription following chronic treatment (Yamada and Higuchi, 2002). Yuan *et al.* (2004)

suggested that a cascade of downstream effects is ultimately responsible for antidepressants' therapeutic effects.

Castrén (2004) suggests that this long-term antidepressant treatment produces molecular effects that resemble those induced by neuronal plasticity and learning. According to Dwivedi *et al.* (2002), several studies suggest that most antidepressants downregulate beta adrenergic and 5-HT_{2A} receptors, but the lack of effects of selective serotonin reuptake inhibitors on 5-HT_{2A} receptors and the upregulation of 5-HT_{2A} receptors by electroconvulsive shock do not explain the therapeutic mechanism of action of antidepressants.

According to Leonard (2003), adaptational changes occur after antidepressant administration in adrenoceptors, serotonin, dopamine and GABA-B receptors. There is evidence that GABA-B receptors play a role in enhancing noradrenaline release in the cortex and in this respect differs fundamentally from the inhibitory GABA-A receptors, which facilitate central GABAergic transmission (Leonard, 2003). A decrease in the activity of GABA-B receptors may therefore contribute to the reduced central noradrenergic tone reported to occur in depression (Leonard, 2003). It is therefore unlikely that dysfunction of a single neurotransmitter system accounts for the action of antidepressants (Dwivedi *et al.*, 2002).

It should be noted that the ultimate function of the nervous system is to store and process information, and this task is not achieved by chemical or molecular changes within individual neurons or synapses, but in the dynamic neuronal networks formed by vast numbers of interconnected neurons. Mood disorders probably imitate compromised function and stability of these networks rather than chemical changes in the brain. Neurotrophic effects of antidepressant drugs could help to restore the functional networks by inducing neurogenesis and facilitating synaptic connectivity. Because these effects take time to arise and mature, it is possible that the antidepressant process is inherently slow (Castrén, 2004).

2.3.2 Anxiety

Anxiety is an unpleasant psychological state accompanied by apprehension, worry, fear, nervousness and sometimes conflict (Leonard, 2003). Arousal is usually heightened (Tardito *et al.*, 2002). An increase in the autonomic sympathetic nervous system is often associated with these psychological changes and may manifest as an increase in blood pressure and heart rate, an erratic respiratory rate, decreased salivary flow leading to dryness in the mouth and throat, and gastrointestinal disturbances (Leonard, 2003). Whilst "physiological" anxiety is usually short-lived, often with a fast onset and sudden end once the aversive event has terminated, "pathological" anxiety occurs when the response of the individual to an anxiety-

provoking event becomes excessive and affects the ability of the individual to lead a normal life (Leonard, 2003).

It has been estimated that 2-4% of the population suffer from pathological anxiety and frequently no contributing factor can be identified (Leonard, 2003).

According to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) classification, anxiety as a disorder can be divided into panic disorder, OCD and social phobia. For the purpose of this study panic disorder and OCD are discussed below.

2.3.2.1 Panic disorder

A panic attack is a discrete episode of unexpected terror accompanied by a variety of physical symptoms (Stahl, 2002).

2.3.2.1.1 Prevalence of Panic disorder

Panic disorder affects up to 2% of the population, but less than one third of these receive treatment. This illness typically begins in late adolescence or early adulthood but can present in childhood and the onset is rare after the age of 45 (Stahl, 2002). Panic disorder is more prevalent in women (Tardito *et al.*, 2002), who have perhaps twice the prevalence of men. Genetic studies demonstrate a 15 to 20% occurrence of panic disorder in relatives of patients with panic disorder, including a 40% concordance rate for panic disorder in monozygotic twins (Stahl, 2002).

2.3.2.1.2 Symptoms

Panic disorder has been regarded as a chronic and recurring illness (Andersch and Hetta, 2003; Kasper and Resinger, 2001).

Associated symptoms include fear and anxiety, as well as catastrophic thinking with a sense of impending doom or the belief that loss of control, death, or insanity is imminent. Physical symptoms can be neurological, gastrointestinal, cardiac, or pulmonary and therefore may mimic many different types of medical illnesses (Stahl, 2002). Behaviours associated with panic attacks typically include an attempt to flee the situation and eventually to avoid anxiety-producing situations or any situation that has previously been associated with a panic attack (Stahl, 2002). Patients with panic disorder run an increase risk of suicidal behaviour (Andersch and Hetta, 2003).

follow-up studies after acute medication treatment indicate that a majority of patients with panic disorder continue to have anxiety symptoms and, although most patients improve, few are cured (Andersch and Hetta, 2003).

Panic disorder is the presence of recurrent unexpected panic attacks followed by at least a 1-month period of persistent anxiety or concern about recurrent attacks or consequences of attacks, or by significant behavioural changes related to the attacks (Stahl, 2002).

2.3.2.1.3 Aetiology

The possible link between panic disorder and major depression has been provided by the frequent occurrence of major depression in patients with panic disorder and agoraphobia, with both conditions responding to antidepressant treatments. However, most of the genetic analyses available suggest that there is an independent genetic basis for these two conditions. (Leonard, 2003).

Panic disorder could involve a dysregulation in the function of several transmitters, including 5HT and NE, GABA, dopamine, acetylcholine and cholecystokinin (Tardito *et al.*, 2002).

One theory of the biological basis of panic disorder is that there is an initial excess of NE. This theory is supported by evidence that panic disorder patients are hypersensitive to α_2-antagonists and hyposensitive to α_2-agonsits (Stahl, 2002). On the other hand, panic patients have a blunted physiological response to postsynaptic adrenergic agonists, perhaps as a consequence of an overactive *l*-NE system. Thus, there may be a dysregulation in the *l*-NE system, with changes in the sensitivity of *l*-NEergic neurons and their receptors, thereby altering their physiological functioning and contributing to the pathophysiology of panic attacks (Stahl, 2002). Together with the emerging biological evidence of NEergic involvement pathophysiology of panic disorder, the hypothesis that NE agents might be uniquely effective in the treatment of panic disorder was suggested. Sasson *et al.* (1999) found evidence for the involvement of NEergic, as well as 5HT neuronal systems, in the pathogenesis of panic disorder. It might be that both neurotransmitters exert their effect through a final common pathway. The study suggests that in panic disorder, the 5HTergic medication is somewhat more effective.

Functional alterations in upstream components of the cAMP signalling as well as lower levels of cAMP have been reported in panic disorder patients. Protein kinase A (PKA) plays a crucial role in the regulation of motor and emotive behaviour, metabolism, learning memory, and stress as well as in the action of psychotropic medications. Patients with panic disorder

have altered levels of platelet PKA. Abnormal levels of PKA may explain, alone or together, with other dysfunctions, some clinical and/or pharmacological features of panic disorder (Tardito *et al.*, 2002).

Various studies suggest α_2-AR dysregulation in panic disorder. Platelet α_2-AR exists in high- and low-conformational states as a function of their coupling to G_i-protein. α_2-AR coupling is important in signal transduction and is modulated by antidepressants (Gurguis *et al.*, 1999).

Evidence suggests that panic disorder may be mediated by dysfunction in the 5HT system, although the role of 5HT in panic is controversial, with some studies suggesting over-activity of 5HT systems, while others suggest under-activity (Kasper and Resinger, 2001). Interestingly, $5HT_{1A}$ agonists, which are endowed with antidepressant properties when given at adequate doses for a sufficient time, have not been found of therapeutic value in panic disorder. Thus, postsynaptic 5HT receptors other than those of 5HT1A are responsible for the anti-panic effect of SSRIs, TCAs and MAOIs (Blier and De Montigny, 1999).

The noradrenergic and serotonergic systems are involved in the pathogenesis of anxiety disorders (Harvey, 2002). Blier and De Montigny (1999) suggested that a hypersensitivity of 5HT and/or NE receptors occurs in patients suffering from panic attacks. 5HTergic neurons have an inhibitory effect on NE neurons in the locus ceruleus and so a 5HT deficit would be expected to result in increased NEergic activity and subsequent autonomic symptoms of panic (Kasper and Resinger, 2001).

The neurotransmitter GABA has also been implicated in the biological basis of panic disorder (Stahl, 2002).

2.3.2.1.4 Treatment

Selective serotonin reuptake inhibitors

The documentation of efficacy for panic disorder and of the safety of these agents has now made them first-line treatments for this condition. Panic patients usually start at a lower dose than depressed patients, due to the supersensitivity of 5HT receptors (Stahl, 2002).

Trycyclic antidepressants

Imipramine (Blier and De Montigny, 1999) and clomipramine have demonstrated efficacy in treating panic disorder. The TCAs have disadvantages that make them second- or third-line

treatments for panic disorder, including anticholinergic side effects, orthostatic hypotension, and weight gain (Stahl, 2002).

Monoamine Oxidase Inhibitors

The classical irreversible MAOIs are effective in treating panic disorder, although disadvantages such as orthostatic hypotension, weight gain, sexual dysfunction, and dietary restrictions, with the potential for a tyramine-induced hypertensive crisis make them second- or third-line treatment for panic disorder (Stahl, 2002).

Benzodiazepines

The Benzodiazepine, alprazolam, has been traditionally used in the treatment of panic disorder (Kasper and Resinger, 2001). Today, Benzodiazepines have become adjunctive treatment to antidepressants, especially for long-term treatment (Stahl, 2002). The habit-forming property of benzodiazepines is well known, and discontinuation of benzodiazepines after maintenance therapy has been reported to produce a withdrawal syndrome (Andersch and Hanna, 2003). Thus, there may be a risk that maintenance therapy with a benzodiazepine, such as alprazolam, might lead to a cycle of dependence, withdrawal, further treatment, and further dependence and abuse (Andersch and Hanna, 2003).

The mode of action of benzodiazepines in alleviating the symptoms of panic disorder has been ascribed to interactions with the GABA system. It has been hypothesized that patients with anxiety disorder have a deficiency in the GABA system, either in reduced receptor sensitivity or a deficit in neurotransmitter (Kasper and Resinger, 2001).

High-potency benzodiazepines are generally more effective in panic disorder than low-potency benzodiazepines (Stahl, 2002).

Myo-Inositol

*m*Ins has been implicated in the treatment of panic disorder, however, the underlying mechanism of action is still not clear (Levine, 1997).

2.3.2.2 Obsessive-Compulsive Disorder

OCD is characterized by obsessions and compulsions severe enough to interfere with a person's ability to function on a daily basis (Kim *et al.*, 2002). Of all the anxiety disorders, OCD may well be the most disabling as well as the least understood (Welkowitz *et al.*, 2000).

2.3.2.2.1 Prevalence of OCD

OCD's lifetime prevalence is 2-3% (Hemmings *et al.*, 2003) and at least 80% of all cases have their initial onset in childhood and adolescence (Rosenberg and Hanna, 2000).

2.3.2.2.2 Symptoms

OCD consists of unusual patterns of behaviour (repetitive behaviours) and highly intrusive and often bizarre ideation (obsessive thoughts) (Welkowitz *et al.*, 2000). The patient experiences obsessions internally and subjectively as thoughts, impulses or images. According to standard definitions in the DSM-IV, obsessions are intrusive and inappropriate and cause marked anxiety and distress (Oken, 2001). Compulsions, on the other hand, are repetitive behaviours or purposeful mental acts that are aimed at reducing anxiety evoked by the intense thoughts. Most often these behaviours are observed by family members or the consulting clinician (Stahl, 2002).

2.3.2.2.3 Aetiology

Some data suggest a genetic component to the aetiology of OCD (Stahl, 2002). According to Leonard (2003), genetic studies lends support to the view that a single gene defect occurs in patients with OCD, while association studies of candidate genes, in spite of methodological difficulties, have highlighted the loci for dopamine 2 (D_2) and dopamine 4 (D_4) receptor genes together with those for catechol-O-methyl-transferase (COMT) and MAOA. Indeed, Rosenberg and Hanna (2000) have stated that OCD is a heterogeneous condition.

Neurobiological abnormalities in the striatum have been hypothesized to be the primary locus of pathology in OCD (Rosenberg and Hanna, 2000). Leonard (2003) has stated that blood flow rates are greater in parts of the cortex in OCD patients than in matched control subjects and that the activity of the inferior prefrontal cortex is significantly greater, than in other brain regions, in untreated OCD patients.

Although it is unlikely that one neurotransmitter system can explain all the complexities of OCD (Stahl, 2002), recent efforts to elucidate the pathophysiology of OCD have centred

largely on the role of 5HT. Among all antidepressant treatments, including ECT, only the drugs that are potent 5HT reuptake inhibitors exert clear therapeutic effect in OCD patients (Blier and De Montigny, 1999). The only neurobiological property that the SSRIs share is their capacity to block the 5HT reuptake process potently, this leaves little doubt that the anti-OCD effect of these drugs is mediated via the 5-HT system (Goodman *et al.*, 1997). Especially given the strong evidence that the abnormalities in OCD are reversed following effective treatment with clomipramine or an SSRI (Leonard, 2003). Despite the convincing evidence that the SSRIs are effective in the treatment of OCD (Stahl, 2002), direct evidence implicating an abnormality in serotonergic function is limited. Enhancement of 5-HT neurotransmission by means of activation of 5-HT_{2A} may be a common feature of many SSRIs (Greenberg *et al.*, 1998). Blier and De Montigny (1999) found that an enhanced 5HT release, resulting from long-term SSRI administration, increases 5HT transmission at postsynaptic 5HT_{2A} receptors. It would appear that while the serotonergic system may be abnormal in OCD, and that drugs, that selectively enhance serotonergic function, are therapeutically effective in treating the condition, the nature of the abnormality in serotonergic function remains unclear.

Up to 60% of OCD patients do not respond to SSRI monotherapy (Stein *et al.*, 1995). Thus, at least some OCD patients fail to demonstrate convincing dysregulation in 5-HT function (Stahl, 2002). Dopamine neurotransmission may be implicated in OCD (Hemmings *et al.*, 2003). It has been established that dopamine plays an important role in reinforcement and the participation of reinforcement on the pathogenesis of compulsive behaviour (Ulloa *et al.*, 2004). In this regard, there is evidence that the dopaminergic system in the basal ganglia is hyperactive in OCD, which may contribute to the main symptoms of the disorder particularly in SSRI resistant patients (Leonard, 2003). It is suggested that the dopamine transmission controls repetitive behaviours (Szechtman *et al.*, 1998) and also plays a role in OCD (Szechtman *et al.*, 1999). The pathology of Tourette's syndrome, which has a significant phenomenological and familial relationship with OCD, is mediated through the dopaminergic system (Pauls *et al.*, 1991).

Both 5-HT and dopamine transmitter systems may be involved in the pathophysiology of the disorder (Stahl, 2002). A number of studies have shown that dopaminergic behaviours are attenuated or reversed by increasing the serotonergic transmission (Ulloa *et al.*, 2004). MAO-A, which is involved in catechol-O-methyltransferase (COMT)-controlled pathways and which is the major enzyme responsible for the degradation of 5-HT, dopamine, NE and epinephrine, has been implicated in mediating OCD (Jenike, 2001). Indeed, D_2 antagonists, such as clozapine, are often effective as adjunctive therapy in SSRI non-responders

(Goodman *et al.*, 1995). This hypothesis is supported by many preclinical data, which suggest that important anatomical and functional interactions exist between 5-HTergic and dopaminergic neurons (Stahl, 2002).

The motor and vocal tics of Tourette's syndrome and other tic disorders can be viewed primarily as neuropsychiatric disturbances with a deficit of motor inhibition at the level of the basal ganglia. These are involved in the feedback loops of cortico-striatal-thalamo-cortical (CSTC) pathways (Rothenberger, 1994). The CSTC circuit, involving glutamatergic and GABAergic inputs from the motor cortex and striatum, regulates motor control. These pathways are modulated by D_1 and D_2 receptors and $5HT_{2A}$ receptors. There appears to be an imbalance of the CSTC circuit in OCD. Stimulation of $5HT_{2A}$ receptors will result in decreased synthesis and release of D, which will modify both GABA and glutamate function (Harvey et al., 2001). Drug treatment (e.g. mainly D_2-blocking agents) decreases the tics and help patients to optimize the central nervous tuning of these circuits to better control their tics (Rothenberger, 1994).

2.3.2.2.4 Treatment

Selective serotonin reuptake inhibitors

SSRIs undoubtedly improve symptoms of OCD, just as they improve symptoms in depression (Stahl, 2002).

Despite the advent of the SSRIs and cognitive behavioural therapy, as many as one third (Rosenberg and Hanna, 2000) to 50% of OCD patients (Goodman *et al.*, 1995) do not respond at all to currently available treatment and many "responders" are only partial responders with continued functional impairment.

However, as compared to the use of antidepressants in depression, a pharmacological response in OCD specifically requires 5-HT and not NE reuptake inhibition. Furthermore, the response to SSRIs in OCD are generally slower, less robust, more likely to relapse after SSRI discontinuation, and not as immediately dependent on synaptic 5-HT availability (Stahl, 2002).

Benzodiazepines

Another pharmacological intervention in the treatment of OCD is the benzodiazepines, which can be combined with a SSRI (Stahl, 2002). The benzodiazepines and structurally related

drugs, act as anxiolytics by activating a specific benzodiazepine receptor which facilitates inhibitory GABAergic transmission.

Atypical antipsychotics

The atypical prototypical clozapine and other novel atypical antipsychotics (AP), such as risperidone, olanzepine, quetiapine and ziprasidone, have shown greater affinity for 5-HT_2 as compared to D_2 receptors and are therefore considered as serotonin-dopamine antagonists (Lykouras *et al.*, 2003). AP, mainly risperidone and olanzepine, have been used as an adjunct therapy in the treatment of resistant OCD (Ramasubbu *et al.*, 2000). There is evidence indicating that some forms of OCD are aetiologically related to Tourette's syndrome, a chronic tic disorder in which traditional neuroleptics (i.e. dopamine antagonists) often produce symptom suppression. The addition of neuroleptics may benefit SSRI-refractory OCD patients who have a comorbid chronic tic disorder (Goodman *et al.*, 1995).

Myo-Inositol

Harvey *et al.* (2001) found that chronic inositol treatment provoked a significant increase in D_2 receptor density. Inositol has proved to be effective in the treatment of OCD (Levine, 1997) however; the mechanism whereby inositol exerts its therapeutic action is still unknown.

Chapter 3 Experimental Procedures

3.1 Experimental Layout

The primary purpose of this study was to investigate the possible modulating role of *myo*-inositol (*m*Ins) on enzymes believed to be involved in antidepressant action. Many antidepressants act via modulating serotonin receptor (5HT-R) action that signals via the phospholipase C-β1 (PLCβ1) pathway. In addition, a recent study in our laboratory (Brink *et al.*, 2004) showed that *m*Ins, fluoxetine and imipramine modulate both $5HT_{2A}$ and muscarinic acetylcholine receptor (mAChR) function, where both of these receptor systems signal via the PLCβ1 pathway. The study therefore focussed on any modulating effects of 24-hour pre-treatment of human neuroblastoma (SH-SY5Y) cells with or without *m*Ins enriched medium on phospholipase Cβ1. In particular it was investigated whether the pre-treatment modulates the mRNA levels for the expression of PLCβ1 and also the expression of PLCβ1 itself. Any modulating effects of *m*Ins on PLCβ1 were then compared with the effects of antidepressant prototypes, such as fluoxetine (FLX), imipramine (IMI) and lithium (LiCl), as well as with that of the cGMP enhancing drug sildenafil (SIL - phosphodiesterase-5 inhibitor). In addition it was investigated whether *m*Ins could enhance any modulating effect of FLX and whether SIL could enhance any modulating effects of *m*Ins.

Other enzymes and proteins were included for optimization of mRNA and protein quantification for future studies. These include the cAMP response element binding protein type 1 (CREB1), glycogen synthase kinase 3β (GSK3β), two subtypes of regulators of G protein-coupled receptor signalling (RGSs), RGS2 and RGS4, three subtypes of Protein kinase C (PKC), PKCα, PKCγ and PKCε, Brain derived neurotropic factor (BDNF) and G-protein receptor kinase 2 (GRK2) for their possible involvement in the mode of action of *m*Ins.

The posttranscriptional investigations (i.e. quantification of mRNA levels after pre-treatments) were investigated by quantitative real time reverse transcription Polymerase Chain Reaction (real time RT-PCR), involving firstly total RNA isolation and then reverse transcription to

complementary deoxyribonucleic acid (cDNA) for amplification with real time RT-PCR. mRNA levels were then compared.

The mRNA levels may not necessarily directly reflect the levels of actual protein produced by the cell, since many factors of regulation occur at the post-transcriptional stage. For posttranslational investigations (i.e. enzyme protein expression), cell membranes of pre-treated cells were prepared and the subcellular fractions were collected and used for Western Blot analyses. The expression levels of the proteins were compared between pre-treatment groups.

Due to the complexity of the study the experimental layout is schematically illustrated in *Figure 1-1*.

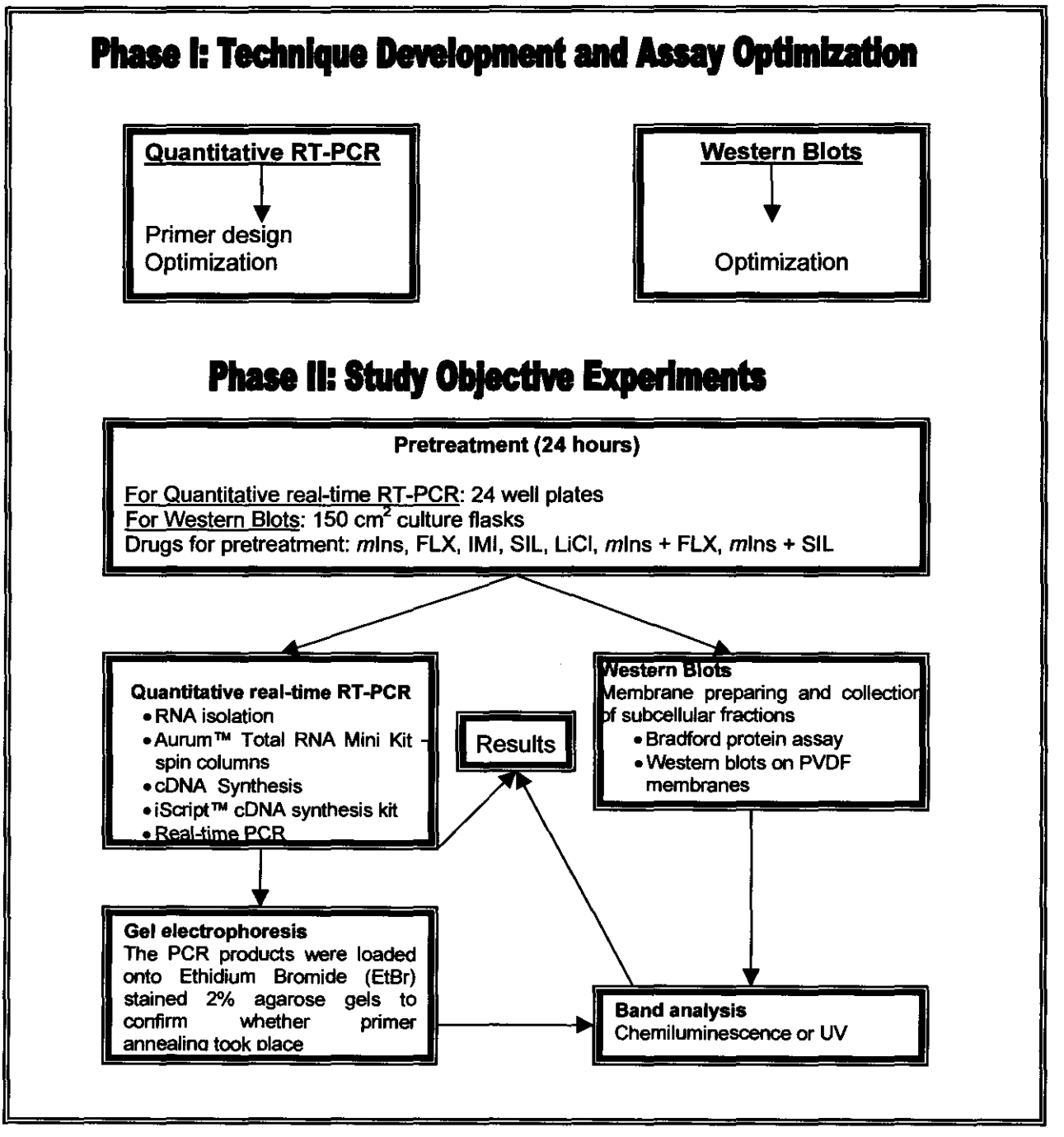


***Figure 1-1:** A schematic layout of the experiments conducted*

3.2 Cell Line

A human neuroblastoma cell line [SH-SY5Y cell line from American Type Culture Collection (ATCC), catalogue number CRL-2266] was used in this study. SH-SY5Y is a thrice cloned (SK-N-SH → SH-SY5 → SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH, which was established in 1970 from a metastatic bone tumor of a 4-year-old girl [American Type Culture Collection (ATCC), 2004]. As it is a neuronal cell line, SH-SY5Y cells were implemented as an *in vitro* biological model of neuronal cells in the central nervous system, where antidepressants exert their primary therapeutic effect.

3.3 Materials

3.3.1 Antibodies

Rabbit polyclonal PLCβ1, rabbit polyclonal pGSK3 beta, mouse monoclonal CREB1 and horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were obtained from Santa Cruz Biotechnologies

3.3.2 Chemicals

myo-Inositol (*m*Ins), imipramine (IMI), sildenafil (SIL), lithium chloride (LiCl), Diethylpyrocarbonate (DEPC), TritonX-100, phenylmethylsulphonyl fluoride (PMSF), aprotinin protease inhibitor, tris(hydroxymethyl)aminomethane (TRIS), ethylenediaminetetra acetic acid (EDTA), acrylamide/bis-acrylamide electrophoresis reagent 40% solution, ammonium persulphate electrophoresis reagent, N,N,N',N-Tetramethylethylenediamine electrophoresis reagent (TEMED), sodium orthovanadate, 2-mercaptoethanol, methanol chromasol V® for high performance liquid chromatography, glycerol and Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) were obtained from Sigma Aldrich (Johannesburg, South Africa). Fluoxetine hydrochloride was a gift from Eli Lilly (Johannesburg, South Africa). Ethanol for molecular biology, sodium chloride (NaCl) and acetic acid glacial were obtained form Merck (Johannesburg, South Africa). β-mercaptoethanol, TRISbase molecular biology grade and Tris/EDTA (TE) buffer 1× molecular biology grade were obtained from Promega (Johannesburg, South Africa / USA). Aurum™ Total RNA Mini Kit, iScript™ cDNA synthesis kit, iQ™ SYBR® Green Supermix, Sodium dodecyl sulphate electrophoresis purity reagent, electrophoresis purity reagent bromophenol blue, glycine electrophoresis purity reagent, Laemmli Sample Buffer, and 10× Tween® 20 solution (polyoxethylene sorbitan monolaurate were obtained from Bio-Rad

Laboratories® (Johannesburg, South Africa). Dulbecco's Modified Eagles Medium (DMEM), Minimum Essential Media with Earle's Base (EMEM), trypsin-versene (0.05% trypsin 1:250 + 0.02% EDTA), and bovine serum albumin were obtained from Highveld Biologicals (Johannesburg, South Africa). Penicillin, streptomycin and amphotericin B were obtained from Bio-Whittaker (Walkersville, MD, U.S.A.). Santa Cruz Western Blotting Luminal Reagent was obtained from Promega (Johannesburg, South Africa). Agarose and Ethidium Bromide were obtained from Separations Scientfic (Johannesburg, South Africa).

3.3.3 Consumables

150 cm^2 culture flasks and 24-well plates were obtained from Corning (New York, U.S.A.). Filtered pipette tips (RNase and DNase free) were obtained from Adcock Ingram (Johannesburg, South Africa). PCR 96-well plates, optical tape and 200 µl thin wall tubes were obtained from Bio-Rad Laboratories® (Johannesburg, South Africa). Immobilon™-P transfer PVDF membranes (pore size 0.45 µM) were obtained from Microcep (Johannesburg, South Africa). Eppendorf 1.5 ml tubes were obtained from Plastpro (Johannesburg, South Africa). Tracker tape was obtained from Amersham Bioscience (Johannesburg, South Africa).

3.3.4 Instruments and Software

Haemocytometer (0.1 mm depth, 0.0025 cm^2), Bio-Rad iCycler iQ detection system, Milton Ray Spectronic 1201 spectrophotometer, Rexall Automatic Speedclave LS-6A autoclave, Sigma 3K15 Beckman centrifuge, Bio-Rad Chemidoc™XRS, Mini-Protean® 3 Cell Western blot apparatus, MSE Sonificator, 96-well plate reader and a 560 nm filter (Labsystems Multiscan RC), Teflon® homogenizer.

The Beacon Designer 3 software was obtained from Bio-Rad Laboratories.

3.3.5 Primer Synthesis

The following internal reverence gene oligos were synthesized by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa): primers for ACTB, HPRT1, HMBS, SDHA and UBC. The primers for the following genes were synthesized by Integrated DNA Technologies, Inc. (IDT) (USA): BDNF, CREB1, GRK2, GSK3 β, PKCα, PKCε, PKCγ, PLC β1, RGS2 (1), RGS2 (2), RGS4 (1) and RGS4 (2).

3.4 Drug Pre-treatments

3.4.1 Seeding of Cells in 24-well Plates

Cells were cultured and maintained in 150 cm^2 culture flasks with DMEM:Ham's F12 medium supplemented with 10% foetal bovine serum (FBS), penicillin 100 units/ml , streptomycin 100 µg/ml and amphotericin B 0.25 µg/ml. Cells were detached from flask bottoms using trypsin versine for 10 minutes. Thereafter, a cell suspension was prepared and the cells were counted by using the haemocytometer. Cells were seeded into 24-well plates at a density of 5 x 10^6 cells per well and were then incubated for five hours in normal growth medium to allow the cells to attach to the well bottoms.

3.4.2 Pre-treatment

3.4.2.1 Pre-treatment Layout

SH-SY5Y cells were treated for 24 hours at 37°C in 5% CO_2 with one of the following drugs in serum-free minimum essential medium, Earle's base (EMEM), enriched with 0.1 mM *m*Ins (normal concentration for growth medium – see § 3.4.2.3), as described by Brink *et al.* (2004).

Table 3-1: *Drugs and the concentrations used in the pre-treatment*

Drug	Concentration
no drug (control)	
*m*Ins	10 mM
FLX	10 µM
IMI	10 µM
SIL	450 nM
LiCl	5 mM
*m*Ins + FLX	10 µM, 10 µM (respectively)
*m*Ins + SIL	10 µM, 450 nM (respectively)

3.4.2.2 Pre-treatment Procedure

The cells in 24-well plates or 150 cm^2 culture flasks were rinsed twice with 500 µl EMEM medium (serum free) to wash away any serum. Thereafter 500 µl of each pre-treatment mixture (*Table 3-1*) was added to the wells and incubated for 24 hours at 37°C in 5% CO_2.

After the 24-hour drug pre-treatment, the cells were rinsed twice with serum-free EMEM and incubated for 18 hours in EMEM + bovine serum to simulate the radio-labelling step described in Brink *et al.* (2004). This produced results for the current studies under comparable conditions to those reported for results on the modulating effect of *m*Ins on $5HT_{2A}$-R and mAChR function.

3.4.2.3 Concentrations Used in Pre-treatment

Myo-Inositol concentrations

DMEM:Ham's F12 medium contains 12.51 mg/l *m*Ins (0.07 mM) and Ham's F12 medium contains 18 mg/ml *m*Ins (0.1 mM). This concentration range can therefore be considered as a normal physiological concentration for cultured cells. Normal brain concentrations of *m*Ins range from 2 to 15 mM and in plasma it is about 5 mg/l (28 µM) (Marcus & Coulston, 1996). Therefore 0.1 mM *m*Ins was used as the standard concentration in control experiments for all pre-treatments. All pre-treatment drugs were added to EMEM with 0.1 mM *m*Ins.

Fluoxetine concentration

FLX has been used in concentrations of 10 µM for *in vitro* studies (Brink *et al.*, 2004; De Kock, 2003; Viljoen, 2002). A concentration of 10 µM FLX represents a high FLX concentration and was used to investigate a maximal modulating effect of FLX.

Imipramine concentration

IMI has been used in a concentration of 10 µM in *in vitro* studies (Brink *et al.*, 2004; De Kock, 2003; Viljoen, 2002; Willets *et al.*, 1996). The peak plasma concentration of IMI after a 200 mg/day oral dose for 4 weeks is 200 ng/ml. IMI is 90.1% protein bound. The free plasma concentration was calculated as 10% of the peak plasma concentration and equals 20 ng/ml = 71 nM. The 71 nM concentration represents a medium concentration. 10 µM represents a high concentration of IMI. Therefore 10 µM IMI was used for the purpose of this study to investigate a maximal modulating effect of IMI.

Sildenafil concentration

The human peak plasma concentration of sildenafil after a single 50 mg oral dose is 212 ng/ml (Thummel & Shen, 2001), which is equal to 446 nM ≈ 450 nM sildenafil (sildenafil citrate MM = 192.122[1]). In a recent study in our laboratory (Eagar, 2004) sildenafil has been shown to exert a significant effect on mAChR function in SH-SY5Y cells at concentrations of 100 and 450 nM. Therefore 450 nM SIL was used for the purpose of this study to investigate a maximal modulating effect of SIL.

Lithium concentration

According to various studies done by Tyobeka & Becker (1998) and Wachira *et al.* (1998), 5 mM LiCl concentration was appropriate to pre-treat cell cultures. Therefore a 5 mM LiCl concentration was used in this study.

3.5 Assays

3.5.1 Quantification of mRNA Levels

3.5.1.1 Total RNA Isolation

Total RNA was obtained from pre-treated SH-SY5Y cells and purified using spin columns from the Aurum™ Total RNA Mini Kit. After pre-treatment of cells in 24-well plates, as described in § 3.4.2.2, the assay was performed following the manufacturer's instructions as follows:

- The culture medium was aspirated and the remaining extra-cellular drug was removed by washing the cells once with 500 µl sterile PBS.
- 500 µl 2-mercaptoethanol was added to the lysis solution (1% final concentration) and 350 µl lysis solution was added.
- The resulting cell suspension was pipetted 12 times up and down to ensure proper lysis of the cells.
- 350 µl ethanol (70%; RNase and DNase free) was added to each well and pipetted up and down to reduce the viscosity.
- RNA binding columns were inserted into 2 ml capless tubes and the lysate transferred to the columns.
- The columns were centrifuged (12 000 x g, 25°C) for 30 seconds in a bench top centrifuge and the filtrate was discarded.

- 700 µl low stringency wash[1] was added
- The columns were centrifuged (12 000 x g, 25°C) for 30 seconds and the filtrate was discarded.
- 250 µl Tris Base (10 mM; molecular grade; pH 7.5) was added to the DNase I powder before initial use.
- 5 µl reconstituted DNase I was diluted with 75 µl for each column and 80 µl diluted DNase I was added to each column.
- The columns were incubated for 15 minutes at room temperature, centrifuged for 30 seconds and the filtrate was discarded.
- 700 µl high stringency wash was added to each column, centrifuged for 30 seconds and the filtrate was discarded.
- The columns were centrifuged for an additional 2 minutes.
- RNA binding columns were placed into 1.5 ml capped tubes.
- 80 µl elution solution (heated to 70 °C) was added to each column and incubated for 1 minute.
- The columns were centrifuged (12 000 rpm, 25 °C) for 2 minutes to elute.
- The eluate containing the RNA was used and the absorbance measured spectrophotometrically at 260 nm. The concentration was determined with the following formula:

$$[\text{RNA}]\,(\mu\text{g/ml}) = \left(\text{Absorbance}^{\lambda 260} - \text{Absorbance}^{\lambda 320}\right) \times \frac{40}{25}$$

3.5.1.2 Reverse Transcription

RNA is single stranded and cannot serve as a template for PCR. Reverse transcription, using the retroviral enzyme reverse transcriptase, was performed to reverse transcribe the RNA template to synthesize complementary DNA (cDNA). Reverse transcription of RNA was carried out with a blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix using the iScript™ cDNA synthesis kit and by following the manufacturer's instructions.

The assay procedure for cDNA synthesis are as follows:

---

[1] The low stringency wash was provided as a 5× concentrate and 80 ml ethanol (95 – 100%) was added to the low stringency wash solution before initial use.

- To obtain a 20 µl product, a master mix of the following reagents was prepared: 5x iScript reaction mix (4 µl per reaction), iScript reverse transcriptase (1 µl per reaction) and nuclease free water (11 µl).
- 4 µl (≈ 1µg) of Total RNA was added to 9 µl aliquots of the master mix.
- Reverse transcription was carried out at 25°C for 5 minutes, then 42°C for 30 minutes and following 95°C for 5 minutes.
- Samples without the enzyme reverse transcriptase were processed in parallel and served as "no template" controls.

3.5.1.3 Quantitative Real-Time RT-PCR

The reverse transcription PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA or DNA. It is the most sensitive method for the detection of low-abundance mRNA. Its application in this study was to compare the levels of mRNA expression after cell pre-treatments. Real Time PCR was performed using the iCycler iQ detection system.

- The PCR reaction was carried out in a 20 µl final volume containing: (1) 10 µl iQ™ SYBR® Green Supermix[2]; (2) 1 µl of 0.5 µM forward and 1 µl of 0.5 µM reverse primers; (3) 2.5 µl cDNA or standard dilution. (4) Water up to 20 µl (sterile and diethylpyrocarbonate-treated (DEPC-treated));
- For each primer pair, a sample without cDNA was included that served as a negative control for DNA contamination. A "no template" control (sample without reverse transcriptase enzyme during reverse transcription) was also included that correlated for the reverse transcription step.
- After an initial denaturation step at 94°C for 3 minutes, temperature cycling was initiated, followed by 40 cycles with denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds (see optimization described in § 3.5.1.6).
- Optical data were collected during the annealing step. A melting curve was generated at the end of every run to ensure product uniformity.

---

[2] SYBR® Green is a double strand DNA binding dye that uses fluorescent dyes that bind specifically to double stranded DNA, containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (Deoxynucleotides) (dATP, dCTP, dGTP and dTTP), iTaq DNA polymerase 50 units/ml, 6 mM $MgCl_2$, SYBR® Green I, 20 nM fluorescein, and stabilizers.

- mRNA in the samples was measured according to the corresponding relative standard curve and the mRNA levels were then expressed in relative concentration normalized against two reference genes. This was achieved by constructing a standard curve for each primer from serial dilutions of cDNA for each PCR run.
- The cDNA concentration was determined spectrophotometrically.

3.5.1.4 Primer Design

Specificity is the most important factor in oligonucleotide primer design for PCR. The primer should only hybridize with the target sequence. The aim was to design oligos for certain target genes that only hybridize with the target sequence in order to quantify these specific protein expressions. When SYBR® Green was used to detect the amplified product, extra caution should be taken in designing the oligos, since its specificity is determined entirely by its primers.

- The Nucleic acid sequence for each gene was downloaded from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).
- The coding region was identified by identifying the initiation codon and the stop codons, and primer design was limited to this region.
- The sequences of the primers were determined using the software Beacon Designer 3.1. Primers were always chosen according to the following parameters: length between 19 and 25 bp[3], Tm comprised between 55°C ± 5°C and length of amplification product between 80 and 170 bp. The designer software limited primer self-complementarity, which could result in formation of secondary structures.
- Manual overview of the electronically designed primers was done and primers were inspected so that the 3' end base was preferably G (guanosine) or C (cytidine) to avoid primer dimers.
- To determine specificity, all sequences were compared with Genbank, using the Blast software available at the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). When both primer sequences showed homology to the same gene, different from that of interest, they were discarded.

[3] The optimal primer length for real-time PCR is between 17-28/15-20 bases in length. When designing an overlong primer, even high annealing temperatures would not be sufficient to prevent mismatch pairing and non-specific priming.

- The following internal reverence genes' sequences were obtained form RTPrimerDB: Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/): Beta actin (ACTB), Hypoxanthine phosphoribosyl-transferase I (HPRT1), Hydroxymethyl-bilane synthase (HMBS), Succunate dehydrogenase complex subunit A (SDHA) and Ubiquitin C (UBC).
- The following sequences were used as described by Larminie *et al.* (2004) and were evaluated by the Beacon Designer 3 and Blast: RGS2 (2) and RGS4 (2).

3.5.1.5 Primer Preparation and Storage

DNA kept frozen in a nuclease-free environment should be stable for years. In addition, precautions should be taken against primer degradation, since inappropriate storage could result in primer degradation, effectively lowering the concentration of primer solutions. To avoid loss of the DNA pellet, the oligos were centrifuged at 1 000 x rpm for five minutes after delivery before opening.

- An EDTA/Tris Buffer molecular biology grade was added to the primers to prepare a 100 µM freezer stock solution.
- Concentrated primers (100 µM) (which should be thawed relatively infrequently) were divided by aliquot into 10 µM working solutions and stored at -86°C.

Only one aliquot for each primer was thawed at a time and stored at -20°C.

3.5.1.6 Optimization for Quantitative Real Time PCR

PCR conditions should be optimized for each primer set prior to use for quantification.

Primer concentration

Too low primer concentrations will result in late amplification or in nonamplification of target sequences; making quantification difficult or impossible. Too high concentrations, on the other hand, may leave large amounts of unused primer that could give rise to non-specific amplification products.

Optimal primer concentrations usually yield amplification with threshold cycle (C_T) values between 10 and 30 cycles. This is usually accomplished by starting off with 150 ng RNA per reaction.

Annealing temperature

The annealing temperature for each primer set was determined using a touchdown program. The touchdown program was started at 2°C lower than the theoretical melting temperature (T_m) of the primer set with the lowest T_m.

During a touchdown program the annealing temperature is progressively lowered by a chosen 0.5°C after the passage of 4 cycles. At some point, the permissive temperature for the primer set will be reached, and amplification of the product will begin. This allows for the determination of the optimal temperature for the primer set and prevents the accumulation of non-specific product and primer dimers.

Melting temperature

The product melting temperature, T_m, was determined by using a melting curve. During a melting curve program, the temperature is increased in a linear fashion, while fluorescence is monitored constantly. At the product's T_m, fluorescence levels rapidly decrease as the product denatures.

The melting curve was examined for linearity and if more than one peak was observed (indicative of secondary products) the temperature conditions were optimized again.

$MgCl_2$ concentration

In general, varying the $MgCl_2$ concentration will result in clean product formation devoid of primer dimers. According to Stagliano et al. (1994), the most common optimal $MgCl_2$ concentration is 4 mM. In this study the iQ™ SYBR® Green Supermix was used which contains 3 mM $MgCl_2$ and therefore no optimization was done on the $MgCl_2$ concentration.

3.5.1.7 Preparation of Diethyl Pyrocarbonate (DEPC) Treated Water

While most sources of fresh, deionized water are free from contaminating RNases, deionized water can be a contributor of RNase activity (destroying sample RNA). Diethyl pyrocarbonate (DEPC) is an RNase and DNase inhibitor, which, at a concentration of about 0.1% (v/v) inactivates RNase, thus protecting RNA against degradation.

- Scott glass bottles and lids were washed with soap and water and rinsed well with water.

- The bottles were rinsed with ethanol to ensure that all soap was removed and were then rinsed thoroughly with ddH_2O to ensure that all ethanol was removed.
- The empty glass bottles were baked for 6-8 hours at 200°C.
- Each bottle was filled with 100 ml ddH_2O
- 100 µl (0.1%) DEPC was added in a fume hood.
- Magnetic stirrers were soaked in 3 % hydrogen peroxide (H_2O_2) and added to the water.
- The solution was stirred slowly overnight to allow any volatile DEPC to escape.
- The bottles were autoclaved at 121°C for 30 minutes to inactivate the DEPC and left undisturbed in the autoclave for 1 hour.
- The stirrers were removed in an RNase-free environment by using a magnet, without touching the water.
- The water was stored at 4°C in a sealed glass container and used for RNA and DNA work only (i.e. opened in an RNase-free environment only).

3.5.1.8 Selection of Reference Genes

Precise quantitative repeatability of RNA isolation and consecutive cDNA syntheses from samples may be difficult, so that it may be difficult to compare mRNA quantity between samples. Accurate normalization of gene-expression levels in a particular sample is therefore an absolute prerequisite for reliable results, especially when the biological significance of subtle gene-expression differences is studied.

The requirements for a proper internal control gene (housekeeping gene) for normalization have become increasingly stringent as knowledge has improved. Importantly, it has been reported that rRNA transcription is affected by biological factors and drugs (Vandesompele *et al.*; 2002). It was therefore important to identify the most stably expressed internal control genes in the SH-SY5Y human neuroblastoma cell line, especially in response to drug pre-treatments.

The purpose of normalization is to remove the sampling differences in order to identify real gene-specific variation. For proper internal control genes, this variation should be minimal or none. A normalization factor based on proper internal control genes should remove all non-specific variation, therefore correcting the sample differences in quality and quantity caused by variations in initial sample amount, variations in nucleic acid recovery, possible RNA degradation of sample material, differences in sample and/or nucleic acid quality, variations in sample loading or pipetting errors and variations in cDNA synthesis efficiency. Vandesompele *et al.* (2002) demonstrated that most non-specific variation was removed

when the most stable control genes were used for normalization. Internal control genes are most frequently used to normalize RNA.

This internal control should not vary in the cells under investigation, or in response to experimental treatment. Importantly, the expression level of the reference gene and the target gene should be comparable, so that the measurements can be done on the same linear scale. This type of quantitative analysis is a common and powerful tool for a highly sensitive determination of RNA expression.

According to Vandesompele *et al.* (2002), the most stable control genes in neuroblastoma cells in ascending order of stability are:

- Beta-2-microglobulin (B2M),
- Ribosomal protein L13a (RPL13A),
- Beta actin (ACTB),
- TATA box binding protein (TBP),
- Tyrosine 3-monooxegenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)
- Hydroxymethyl-bilane synthase (HMBS),
- Ubiquitin C (UBC),
- Succunate dehydrogenase complex, subunit A (SDHA),
- Hypoxanthine phosphoribosyl-transferase I (HPRT I) /Glyceraldehyde-3-phosphate dehydrogenase (GAPD).

ACTB, HMBS, HPRT1, SDHA and UBC internal controls were selected/unselected based on the following:

- GAPD, although the most stable in neuroblastoma cells, is documented to have variable mRNA levels. In addition GAPD contributes to diverse cellular functions such as DNA replication and it is implicated in apoptosis and neurodegenerative diseases.
- Due to the fact that the expression level of the target gene and that of the reference gene should be in the same range and since the expression levels of the target genes were not known, a range of reference genes with different expression levels were chosen. The expression levels of these selected reference genes ranging from low to high are as follows: HMBS < HPRT1 < SDHA < ACTB < UBC.

3.5.2 Quantification of Protein Expression

3.5.2.1 Membrane Preparation and Subcellular Fractionation

Some proteins, such as PLCβ1, are membrane-bound, while others, such as CREB1 occur in the nuclei. To determine protein expression in cell membranes, membranes were prepared from pre-treated cells and the cytosol fraction (nucleus and larger organelles) was also prepared from the same cells by collecting the pellet after centrifugation at lower speed. Both the cytosol and membrane fractions were used in the Western Blot assay for specific protein quantification.

- After the drug pre-treatments in 150 cm^2 flasks (5 confluent flasks per pre-treatment group, as described in § 3.4.2.2), the medium was aspirated and the cell monolayers were loosened with Trypsin/Versin.
- Cell scrapers were used to ensure that all cells were detached from the flask surfaces
- 10 ml PBS was added to each flask and pipetted up and down to break up cell clusters.
- The cells were placed in 50 ml conical tubes and centrifuged in the Sigma bench top centrifuge at 5 000 rpm and 4°C for 10 minutes.
- The supernatant liquid was discarded and the washing procedure above, comprising of the addition of 10 ml PBS, followed by centrifugation, was repeated twice.
- The supernatant liquid was discarded and the cell pellet was re-suspended in 20 ml of 1 mM Tris Buffer (pH 7.4; 4°C).
- These tubes with the cell suspension were tumbled at 4°C for 15 minutes.
- The suspension of undisrupted cells were homogenized five times with a Teflon homogenizer, whereafter the suspension had no visible particles.
- The suspension was centrifuged at 1000 x g for 10 minutes at 4°C
- The supernatant liquid was collected and the pellet was again resuspended in 10 ml of 1 mM Tris Buffer (pH 7.4; 4°C), tumbled, homogenized and centrifuged.
- The pellet (containing the cell nuclei and larger organelles) was sonicated and transferred to a 1.5 ml eppendorf tube (CYTOSOL FRACTION).
- The resulting supernatants (containing the membrane) were centrifuged in the ultracentrifuge at 40 000 x g for 60 minutes at 4°C.

- The membrane-containing pellet was re-suspended in 50 µl of a lysis buffer[4], sonicated and transferred to a 1.5 ml eppendorf tube (MEMBRANE FRACTION).
- The tubes were snap-frozen in *l*-N_2 and stored at -80°C.
- Both the membrane and cytosol fractions were used to determine the protein expression by using Western Blot analysis.

3.5.2.2 Determination of Protein Concentration

The protein concentration in both the membrane and cytosol fraction samples were determined to ensure the loading of equal protein quantities onto the Western Blot gels, thereby making relative quantification of protein expression between treatment groups possible. The Bradford assay was performed as follows:

- 2 mg bovine serum albumin (BSA) was weighed and dissolved in 1 ml double distilled water.
- Protein standards were prepared with the BSA in the following concentrations: 0.1 mg/ml, 0.4 mg/ml, 0.7mg/ml, 1.0 mg/ml and 1.4 mg/ml.
- 5 µl of each protein standard, the cell membrane and nuclei samples and lysis buffer were added to a 96-well plate in duplicate.
- A blank sample, containing only double distilled water, was also added.
- 250 µl Bradford reagent was added to each well and immediately mixed in a plate shaker for 30 seconds.
- Thereafter the plate was incubated for 15 minutes at room temperature.
- The absorbance was determined at 560 nm.
- The average blank absorbance value was subtracted from all other absorbance values.
- The net absorbance was plotted against protein concentration and the unknown protein concentrations were determined.

3.5.2.3 Western Blot

To quantify protein expression (i.e. to determine posttranslational changes after drug pre-treatments), Western blot analyses were performed on the membranes prepared from pre-reated SH-SY5Y cells. The assays were performed as follows:

---

[4] The 10× lysis buffer contained the following reagents: 100 mM Tris, ph 7.4, 10% SDS in water.

Table 3-2: *Buffers and gel formulations*

	Reagent
Resolving gel	8% acryl/bisacrylamide
	1.5M Tris-HCl (pH 8.8)
	10% SDS
	10% Ammonium persulphate
	0.005% TEMED
	Double distilled water (ddH_2O)
Stacking gel	3.9% acryl/bisacrylamide
	0.5M Tris-HCl, (pH 6.8)
	10% SDS
	10% Ammonium persulphate
	0.25% TEMED
	ddH_2O
Running Buffer	25 mM Tris Base
	192 mM Glycine
	3.5 mM SDS
Loading Buffer	0.5 MTris-HCl
	92.09 M Glycerol
	10% SDS
	0.5% bromophenol blue
	78.13 M 2-Mercaptoethanol
	ddH_2O
Transfer Buffer	25 mM Tris
	192 mM glycine
	20% methanol
	0.05% SDS
	ddH_2O
Tris Buffered Saline-Tween (TBS-T) Buffer	10 mM Tris
	100 mM NaCl
	10% Tween
Blocking Buffer	TBS-T Buffer
	5% fat free milk powder

- 8% polyacrylamide resolving gels were prepared and poured into two gel cassettes.
- The solution was immediately overlaid with water and the gel was allowed to polymerize for 40 minutes.
- The gel surface was then rinsed with distilled water.
- Two 3.9% polyacrylamide stacking gels were prepared.
- The stacking gels were allowed to polymerize for 30-40 minutes.
- A comb was inserted between the spacers.
- The comb was removed gently and the wells were rinse thoroughly with running buffer.
- The gel cassette assemblies were removed from the casting stand and placed first into the electrode assembly and then into the clamping frame and finally into the Mini tank.
- The protein samples were diluted with loading buffer for loading.
- Equal amounts of protein (4 ug per lane) and a marker were loaded into separate wells, using a micropipette with loading tips.
- The tank was filled with running buffer.
- After one hour of electrophoresis at 100 volts, the gels were removed and soaked in transfer buffer for 15 minutes.
- The membranes and filter paper were cut to the dimensions of the gel.
- The membranes (wetted in methanol), filter paper and fibre pads were soaked in the transfer buffer for 15 minutes.
- The gels were transferred to the filter paper and the PVDF membranes were positioned onto it.
- The proteins were electrophoretically transferred to the membrane for two hours at 0.08 A.
- The membranes were removed and blocked overnight in a blocking buffer.
- The membranes were washed every 5 minutes for 30 minutes to remove all the blocking buffer and the primary antibody was added to blocking buffer.
- Each membrane was probed with different antibodies, against the PLC β1 isozyme,, CREB1 or GSK3 β and was incubated with a 1:2 000 (1 µl/ml), 1:2 000 (1 µl/ml), and 1:1 000 (2 µl/ml), respectively primary antibody for one hour.
- The membranes were washed again in TBS-T buffer every five minutes for 30 minutes to remove all primary antibody.
- The secondary antibody solution was prepared in blocking buffer at a concentration of 1:4 000 (0.25 µl/ml) and the membranes were incubated for one hour.

- The membranes were washed again every 5 minutes for 30 minutes to remove all unbound secondary antibody.
- Fluorescence tape was cut and pasted onto the visible marker.
- Detection was performed with a Promega ECL system.
- ECL Solution A and B were mixed in a ratio of 1:1 and the membranes were incubated with 1 ml for 10 seconds in the darkroom.
- The membranes were wrapped in cling wrap and exposed to X-ray film.
- The intensity of bands was quantified by using a UV transilluminator.

3.5.2.4 Gel Electrophoresis

The aim of this experiment was to confirm whether primer annealing took place. The PCR products were loaded onto ethidium bromide (EtBr) stained 2% agarose gels and the bands were analysed.

Table 3-3: *Buffer formulations*

Tris-Acetate-EDTA (TAE) Buffer	40 mM Tris Base
	20 mM Glacial acetic acid
	1 mM EDTA (pH 8.0)
	ddH_2O
Loading dye	50% Glycerol
	0.25% bromophenol blue
	TAE buffer

- The amount of agarose required for preparing the desired agarose gel concentration and volume were determined and added to 80 ml of 1 x TAE electrophoresis buffer.
- This solution was boiled until all the agarose was dissolved and then cooled down until ± 45°C was reached.
- A 1% EtBr solution was added to the agarose solution.
- A removable tray (UVTP) was put into a mini-gel caster and the comb was placed into the appropriate slot of the tray.
- After the solution was poured into the tray and allowed to solidify, the comb was removed and the gel was placed into the Wide Mini-Sub cell GT system.
- The sample loading dye was added to the samples to make samples dense for under laying into the sample wells.

- The samples were loaded into the wells using standard pipettes and a 1 kbp DNA ladder molecular weight marker was run on every gel to confirm expected molecular weight of the amplification products.
- After 30 minutes of electrophoresis at 100 volts, the gel was placed on a UV transilluminator for nucleic acid visualization and analysis.

3.6 Data Analysis

3.6.1 Quantitative Real-Time RT-PCT Data Analysis

Data from assays were obtained as duplicate measurements from at least two individual, comparable experiments.

The target gene[5] (PLCβ1 and GSK3β) concentration in each sample was calculated relative to the internal reference gene and the result was expressed as a target/reference ratio. The following formula was used to calculate relative expression levels:

$$\text{Normalized ratio} = \frac{\text{sample concentration}}{\text{reference concentration}} : \frac{\text{control target concentration}}{\text{control reference concentration}}$$

Equation 3-1: *Relative expression levels by using normalization*

The relative concentration of the target gene and the reference gene were determined using a relative standard curve. The relative ratio of target to reference for each sample and for the control group is calculated in the first step[6]. The target/reference ratio for each sample was then divided by the target/reference ratio of the control group[7]. Thus the normalization to a control provides a constant calibration point between PCR runs.

---

[5] See § 3.5.1.8

[6] This corrects for sample-to-sample variations caused by differences in the initial quality and quantity of the nucleic acid.

[7] This second step normalizes for different detection sensitivities of target and reference amplicons caused by different probe annealing, quantum yields of dye batches or SYBR® Green efficiency

3.6.2 Western Blot Data Analysis

Data from assays were obtained from at least three individual experiments and the intensity of bands was quantified by using an imager system. The intensity of the bands was compared and statistically analysed as explained in § 3.6.3.

3.6.3 Statistical Analyses

All data comparisons between treatment groups were performed by the Statistical Services of the North-West University (PUK). Data were tested for statistical differences implementing the ANOVA and Tuckey post-test. The student-t test and one sample-t test was used to test for differences from control.

Chapter 4 Results and Discussion

In this chapter the results of the experiments performed will be presented and discussed.

4.1 Protein Expression

4.1.1 Membrane Protein Concentrations

The protein concentrations of the membrane and the cytosol fractions were determined by using the Bradford protein Assay. The BSA standard curve is presented in Figure 4-1:

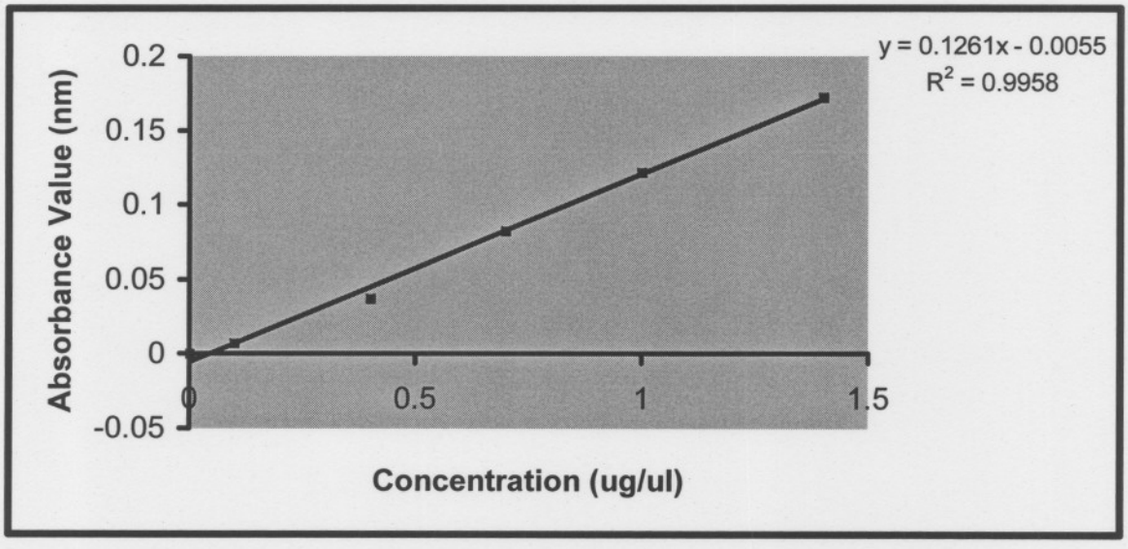


Figure 4-1: *BSA standard curve from Bradford protein assay*

It can be seen in Figure 4-1 that the standard curve is linear and valid in a concentration range of 0.1 µg/µl to 1.4 µg/µl.

In the following Table (4-1) the absorbance values and concentrations of the proteins of the membrane and the cytosol fraction can be seen:

Table 4-1: *(A) Protein concentrations of the cell membrane fraction. (B) Protein concentrations of the cell cytosol fraction*

(A)

Membrane Fraction		
Drug Pre-treatment	***Absorbance[1] at 560 nM***	***Concentration (µg/µl)***
Control	0.96	0.25
*m*Ins	0.31	0.19
FLX	0.99	0.33
IMI	0.63	0.34
SIL	0.78	0.51
LiCl	0.51	0.12
*m*Ins + FLX	0.89	0.38
*m*Ins + SIL	2.37	0.37

(B)

Cytosol Fraction		
Drug Pre-treatment	***Absorbance[2] at 560 nM***	***Concentration (µg/µl)***
Control	0.25	0.96
*m*Ins	0.19	0.31
FLX	0.33	0.99
IMI	0.34	0.63
SIL	0.51	0.78
LiCl	0.12	0.51
*m*Ins + FLX	0.38	0.89
*m*Ins + SIL	0.37	2.37

The absorbance values (Table 4-1) were used to calculate protein concentration. The concentration of the membrane fraction proteins varies between 0.119 µg/µl and 0.38065 µg/µl. The concentration of the nucleus and organelle fraction proteins varies between 0.305 µg/µl and 2.369 µg/µl. Due to the variation in protein concentration between the different

[1] Absorbance values presented have been corrected for background.

[2] Absorbance values presented have been corrected for background.

drug pre-treatments, it can be assumed that some drug treatments (like lithium) detach cells from the bottom of the cell culture flasks. Equal quantities (4 µg) of protein for each drug series were loaded onto the gels for the Western Blot assay.

4.1.2 Western Blot Results

4.1.2.1 Phospholipase C β1 (PLCβ1) Western blot results

PLCβ1 Cytosol protein Fraction

The Western blot results for PLCβ1 in the cytosol protein fraction is presented in Figure 4-2 below:

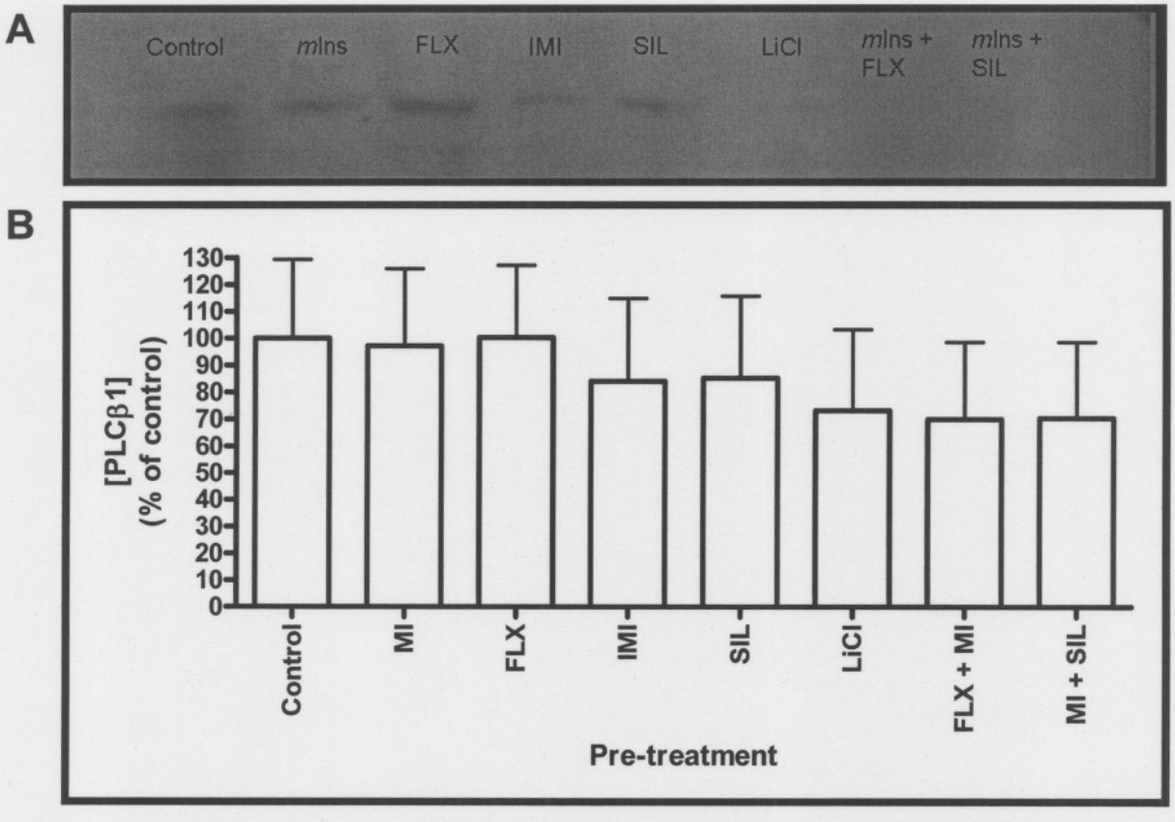


Figure 4-2: *(A) Representative Western Blots of PLCβ1 as found in the cytoplasmic fraction of human neuroblastoma SH-SY5Y cells before and after 24-hour pre-treatment with the indicated drugs. Please note that all three experiments yielded comparable results. (B) Bar chart of the relative concentrations of PLCβ, as determined from (A)and corrected for background . All data are averages of single observations from three independent experiments, expressed as percentage of control ± standard error of the mean (SEM).*

The bands for PLCβ1 (at 150 kDa) can be seen in Figure 4-2(A). Although the visual presentation of this figure suggests a decrease in PLCβ1 concentration after LiCl, *m*Ins + FLX and SIL + *m*Ins, analyses by the transilluminator, as presented in Figure 4-2(B) reveal that there are no significant quantitative difference in the intensity of the bands.

PLCβ1 Membrane Fraction

The Western blot results for PLCβ1 in the membrane of human neuroblastoma cells are showed in Figure 4-3 below:

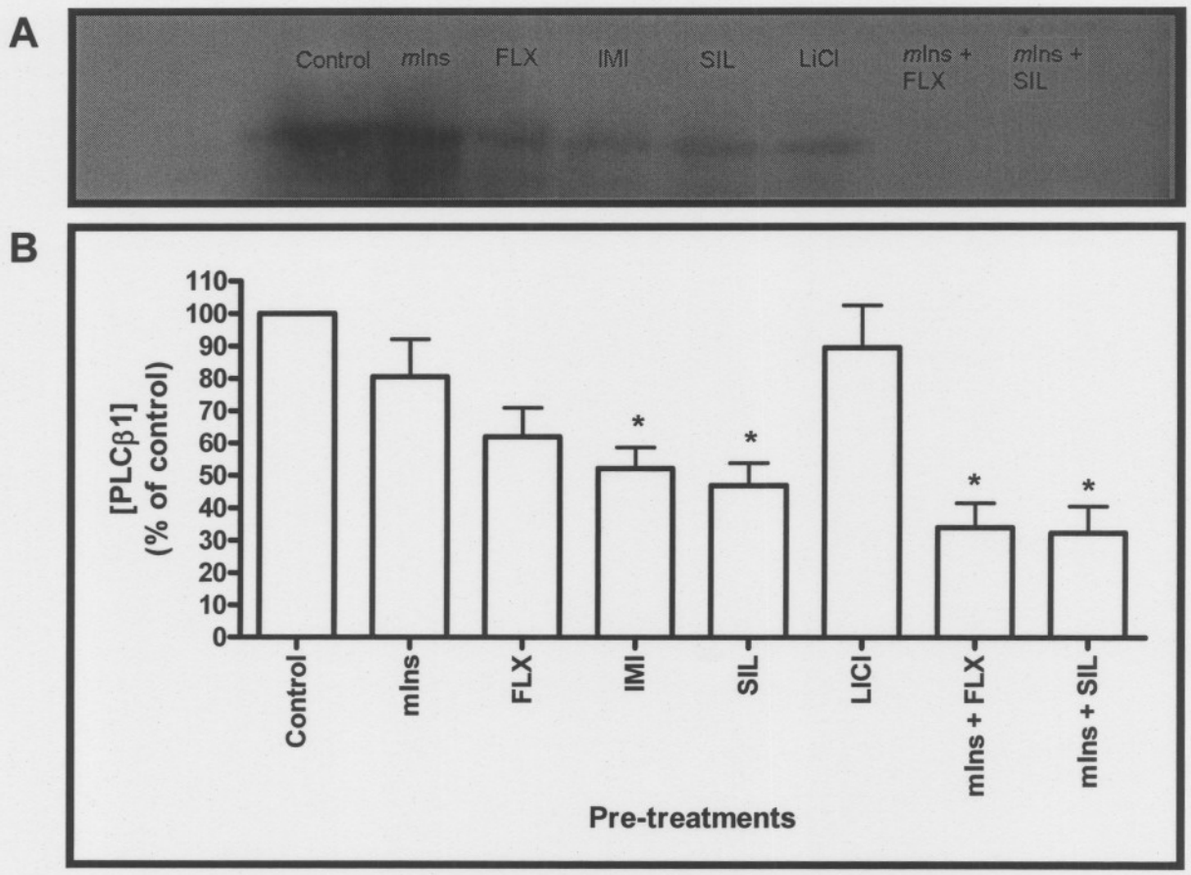


Figure 4-3: *(A) Representative Western Blots of PLCβ1 as found in the membrane fraction of human neuroblastoma SH-SY5Y cells before and after 24-hour pre-treatment with the indicated drugs[3]. Please note that all three experiments yielded comparable results. (B) Bar chart of the relative concentrations of PLCβ, as determined from (A)and corrected for background. All data are averages of single observations from three independent experiments, expressed as percentage of control ± standard error of the mean (SEM).*

Figure 4-3 depicts that all drug pre-treatments, except LiCl, decreased the PLCβ1 concentration in the membrane fraction. However, the small decreases seen with *m*Ins or fluoxetine alone were not statistically significant. The relative concentration values, as displayed in Figure 4-3, are given in Table 4-2 below:

The following table contains the average intensities of the membrane PLCβ1 after the indicated drug pre-treatments:

[3] Due to limited sample material, only 50% (LiCl) and 75% (*m*Ins) of the amount of protein (4 µg) was loaded onto the gel. The intensity values were corrected.

Table 4-2: *The one-sample Student t-test[4] performed on the Western blot results of the PLCβ1 membrane fraction, to determine whether the treatment values differed significantly from control.*

Drug Pre-treatment	*Mean Average Relative [PLCβ1] (% of control)*	*SEM*	*n*	*p-value*
Control	100.0	0	3	-
*m*Ins	76.4	10.4	3	0.23
FLX	58.7	7.7	3	0.05
IMI	50.4	9.5	3	0.02
SIL	45.2	8.7	3	0.02
LiCl	88.5	23.4	3	0.50
*m*Ins + FLX	35.3	14.1	3	0.01
*m*Ins + SIL	33.8	14.4	3	0.01

In Table 4-2 the data are presented as averages of single observations from three independent experiments, expressed as percentage of control ± standard error of the mean (SEM). The one-sample Student t-tests shows a marked decrease in the expression of PLCβ1 after the following drug pre-treatments: IMI ($p = 0.02$), SIL ($p = 0.02$), *m*Ins in combination with FLX ($p = 0.01$) and SIL ($p = 0.01$) respectively.

The following table contains the results of the Tukey HDS posttest (after one-way ANNOVA) that has been performed on the intensities of the PLCβ1 membrane fraction bands to detect significant differences between the bands:

[4] The Dunnett's posttest could not be performed, since no standard error was available for the 100% control values.

Table 4-3: *The p-values from a statistical analysis of the PLCβ1 membrane fraction: Tukey HDS posttest*

	mIns	*FLX*	*IMI*	*SIL*	*LIT*	*mIns +FLX*	*mins + SIL*
mIns		0.78	0.36	0.20	0.99	**0.04**	**0.03**
FLX	0.78		0.99	0.90	0.40	0.40	*0.32*
IMI	0.36	0.99		1.00	0.13	0.80	0.73
SIL	0.20	0.90	1.00		0.07	0.95	0.91
LiCl	0.99	0.40	0.13	0.07		**0.01**	**0.01**
mIns + FLX	**0.04**	0.38	0.80	0.95	**0.01**		1.00
mIns + SIL	***0.03***	0.32	0.74	0.91	**0.01**	1.00	

In Table 4-3, the statistical differences of the bands of PLCβ1 after the drug pre-treatments can be seen at a p-value < 0.5. There was a marked difference between *m*Ins pre-treatment and the *m*Ins in combination with FLX (p = 0.04) and SIL (p = 0.03) respectively.

However, the small decreases in PLCβ1 concentration seen with *m*Ins or fluoxetine pre-treatment alone were not statistically significant. Repeats of the experiment (i.e. increase in "n"), may potentially yield results with a statistically significant difference from control, but this is speculative. A combination of FLX + *m*Ins, however, yielded a statistically significant decrease in the PLCβ1 concentration. Brink *et al.* (2004) found that both *m*Ins and FLX pre-treatments (similar to the current study) decreased muscarinic acetylcholine receptor (mAChR) and serotonin 5-HT_{2A} receptor (5-HT_{2A}-R) function. These receptors both signal via G_q proteins to activate PLCβ1 (Offermanns, 2003). The results from the current study, i.e. the reduction in PLCβ1 after FLX + *m*Ins, would therefore provide one explanation for the results observed by Brink *et al.* (2004).

Figure 4-3(B) depicts that imipramine (IMI) decreased the PLCβ1 concentration statistically significantly. According to Brink *et al.* (2004) IMI reduces mAChR function and increases 5-HT_{2A}-R function. This inhibitory effect of IMI pre-treatment on PLCβ1 could possibly explain the reduction in mAChR function, but not the increase in 5-HT_{2A}-R function. PLC-β1 is primarily activated by G_q proteins, which couple to receptors such as 5-HT_{2A}-Rs, 5-HT_{2C}-Rs, α_1-ARs and mAChRs (Dwivedi *et al.*, 2002). However in a study done by Dwivedi *et al.* (2002), chronic antidepressant treatment (desimipramine, fluoxetine) decreased PI-PLC activity and specifically decreased the protein level of PLC-β_1 isozyme in membrane and

cytosol fractions of cortex and hippocampus of male rats. These findings suggest that that antidepressants regulate the transcription of specific PLC-β_1 isozyme and that altered PI-PLC activity could be related to alterations in the expression of this specific PLC isozyme (Dwivedi *et al.*, 2002). This could possibly explain the reduction of PLCβ1 in the membrane fraction.

According to the results in Figure 4-3(B), SIL inhibits PLCβ1 expression. In addition, there is a trend for SIL to potentiate the inhibitory effects of *m*Ins on PLCβ1 expression, although the difference did not reach statistical significance. It is therefore not clear if this potentiation is due to the effect of SIL on its own or in combination with *m*Ins. Unpublished data (Eager, 2004) showed that a 24-hour pre-treatment of neuroblastoma SH-SY5Y cells with SIL (similar to the current study) potentiates mAChR function. This potentiating effect of SIL is seemingly contradictory to the reduction in PLCβ1 concentration observed after SIL pre-treatment (as seen in Figure 4-3), so that the results from the current study cannot explain the results observed by Eagar (2004).

4.1.2.2 Western blot results for p-GSK3β

The Western blot results for phosphorylated glycogen synthase kinase 3β (p-GSK3β) after the pre-treatment with the different drugs are shown below:

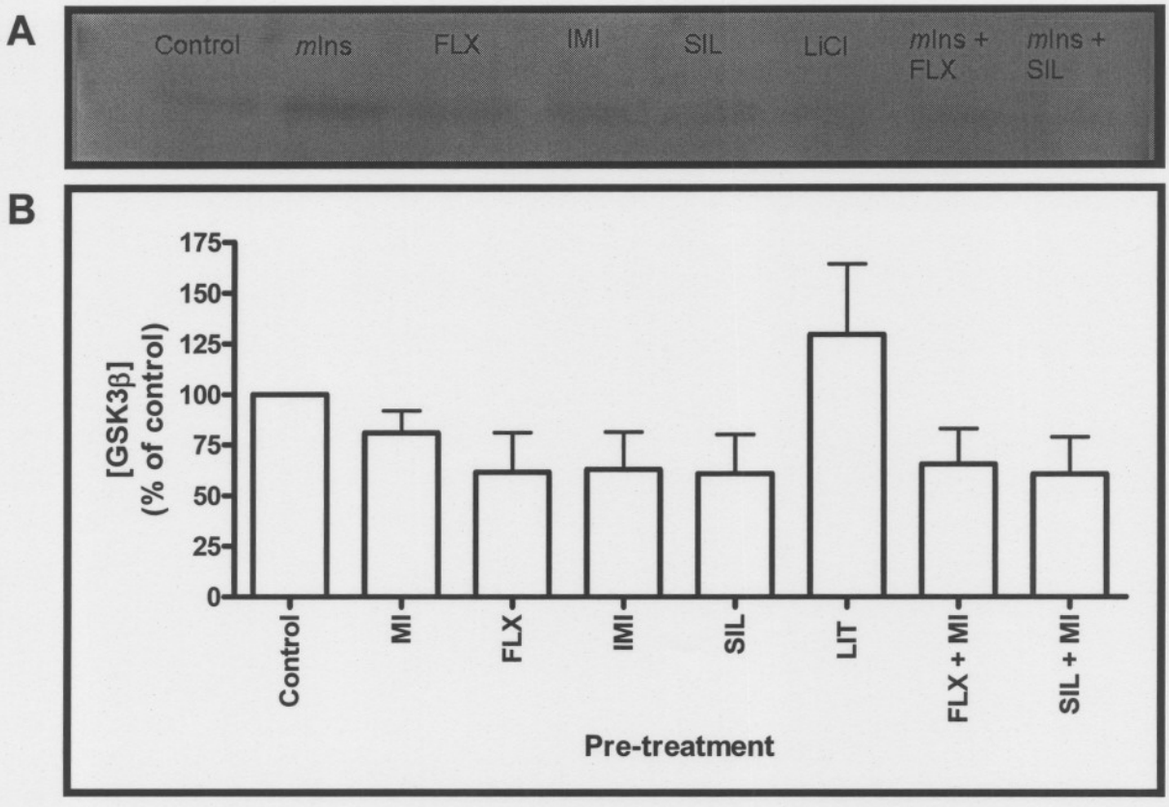


Figure 4-4: *(A) Representative Western Blots of GSK3β (46 kDa) as found in the membrane fraction of human neuroblastoma SH-SY5Y cells before and after 24-hour pre-treatment with the indicated drugs. Please note that all three experiments yielded comparable results. (B) Bar chart of the relative concentrations of GSK3β, as determined from (A)and corrected for background. All data are averages of single observations from three independent experiments, expressed as percentage of control ± standard error of the mean (SEM).*

The Western blot analysis of p-GSK3β (Figure 4-4) show that the protein levels of p-GSK3β in the cytosol or membrane fractions before and after the pre-treatments with the indicated drugs did not reach statistical significance[5]

LiCl treatment inhibits GSK3β and this effect may possibly contribute to the neuroprotective properties of lithium (Jope, 2004). However, i*n vitro* studies demonstrated that lithium's neuroprotection is both time-dependant and dose-dependent, and apparently relevant to its therapeutic index in clinical application (Yuan *et al.*, 2004). This neuroprotection is long

[5] For statistical analysis the one-sample student's-t tests were performed to compare drug pre-treatments with the control (i.e. whether values differed statistically significantly from 100%). No statistically significant differences were found between the control and any of the pre-treated groups.

lasting, occurs at therapeutically relevant concentrations of lithium, and requires treatment for 6-7 days in cell cultures (i.e. *in vitro*) for complete protection to occur. In contrast, a 24-hour treatment with lithium may be ineffective (Nonaka *et al.*, 1998). Therefore, a longer pre-treatment of 6-7 days with concentrations of 1.2 mM or a higher concentration of 3 mM lithium, led not only to loss of the neuroprotective effects but also to a neurotoxic effect (Nonaka *et al.*, 1998). On the other hand, Becker and Tyobeka (1998) used a 5 mM (growth-enhancing concentration) of LiCl concentration in cell cultures for it did not modulate the intracellular concentrations of cAMP, inositol, inositol monophosphate, inositol biphosphate and IP_3. Wachira et al. (1998) used LiCl for the treatment of cell cultures at a "low" concentration of 5 mM and reported that a 10 mM (and above) LiCl concentration induced apoptosis. This may explain why no inhibition of GSK3β by lithium pre-treatment is seen in Figure 4-5.

4.1.3 Primer design for Quantitative real time RT-PCR

Genbank blasts for the sequences of the human mRNA for the expression of the indicated proteins were performed as described in §3.5 4. Results were obtained as given in Table 4-4.

Table 4-4: *mRNA Sequences and Blast results of sequences of genes for the expression of various proteins. Usually multiple submitted sequences for each gene were found, where criteria for the final choice of a sequence included complete sequences over sequences for a section of the gene only and sequences for total, over for isoform- or variant specific protein expression (unless when a specific isoform was preferred).*

Protein[6]	Number of Hits	Sequence Chosen (accession no.)	Definition	Reason for Choice
BDNF	78	AF400438	Homo sapiens brain-derived neurotrophic factor, mRNA	This sequence contained the complete coding sequence (cds) for total BDNF, while some of the other sequences were for specific isoforms only.
CREB1	9	NM_004379	Homo sapiens	This sequence contained the complete coding sequence (cds)
GSK3β	7	NM_002093	Homo sapiens glycogen synthase kinase 3 beta, mRNA	Other sequence specified transcript variants and not the total GSK3β
PKCα	372	NM_002737	Homo sapiens protein kinase C alpha, mRNA	This sequence was without transcript variants, while some others were for specific isoforms
PKCε	372	NM_005400	Homo sapiens protein kinase C epsilon, mRNA	This sequence was without transcript variants, while some others were for specific isoforms
PKCγ	372	NM_002739	Homo sapiens protein kinase C gamma, mRNA	This sequence was without transcript variants, while some others were for specific isoforms
PLCβ1	79	BC069420	Homo sapiens PLC beta 1 (phosphoinositide specific), transcript variant 1, mRNA	This sequence contained the complete CDS, while some others were for specific isoforms
RGS2	53	NM_002923	Homo sapiens regulator of G-protein signalling 2, mRNA	This sequence was without transcript variants, while some others were for specific isoforms
RGS4	53	NM_005613	Homo sapiens regulator of G-protein signalling 4, mRNA	This sequence was without transcript variants, while some others were for specific isoforms

After an appropriate human mRNA nucleic acid sequence for each gene was obtained and the coding region was identified, the sequence was entered into the Beacon designer 3 software and primers were designed according to specified criteria (§ 3.5.1.4).

---

[6] Brain derived neurotropic factor (BDNF), cAMP response element binding protein type 1 (CREB1), glycogen synthase kinase 3β (GSK3β), Regulators of G protein-coupled receptor signalling (RGSs), RGS2 and RGS4, Protein kinase C (PKC), PKCα, PKCγ and PKCε.

Specificity was determined by comparing the sequences with Genbank, using the program Blast that is available at the National Centre for Biotechnology Information (NCBI) website. The Blast results for the various oligos are shown in Appendix B.

Databank searches and analyses of the following primer oligo sequences, as shown in Table 4-5 below, revealed no homology with other known genes. Chosen primers also had low "E-values"[7] (expect values), indicating high selectivity and specificity. These primers are therefore suitable for specific recognition of the intended protein only. Twelve genes that have been implicated in psychiatric disorders were chosen and the primers for these genes were designed and synthesized. After optimization, the best optimized primer was chosen in order to perform real-time RT-PCR. The designed oligo sequences are presented in Table 4-5:

Table 4-5: *Oligo primer sequences of target genes as chosen and synthesized*

Oligo	*Forward primer*	*Reverse primer*
BDNF	TTG ACA TCA TTG GCT GAC ACT TTC G	AGG CAC TTG ACT ACT GAG CAT CAC
CREB1	GGA GCC GAG AAC CAG CAG AG	AGA GTT ACG GTG GGA GCA GAT G
GRK2	AGC GAT AAG TTC ACA CGG TT	TGC CAC CGC TCC GAG AGT GTG A
GSK3β	GCA CTC TTC AAC TTC ACC ACT CAA G	TGT CCA CGG TCT CCA GTA TTA GC
PKCα	CAG GGA GAT CCA GCC ACC ATT C	CTG TCC TCG TGT GAA GAA CTT GTC
PKCε	GTT CGG TAT CCA CAA CTA CAA GGT C	TGG CGA TTC CTC TGG CAT CC
PKCγ	CGG CGT AGG CGA TTC AGA GG	GAA GAA GCG AGC GGT GAA CTT G
PLCβ1	AGA ACA AGC CTC CAA CAC CTA CAG	CAT CAT CAT CAT CGT CGT CGT CAC
RGS2 (1)	AGT GCT ATG TTC TTG GCT GTT CAA C	TTC TCG CTT CTC CTC GCT CTT G
RGS2 (2)	TTC TGG CTG GCC TGT GAA G	GCA GTT GTA AAG CAG CCA CTT G
RGS4 (1)	TCA GTC CAG GCA ACC AAA GAG G	GAG GAA GCG GCG GTA GGA ATC
RGS4 (2)	ACC AGG GAA GAG ACA AGC CG	ACT TGA GGA AGC GGC GGT AG

For RGS2 and RGS4, two primer sets each were designed. Primer sequences for RGS2(2) and RGS4(2) were used as described by Larminie *et al.*, (2004), and G protein-coupled

[7] E-value describes the random background noise that exists for matches between sequences. The lower the E-value, the more "significant" is the match, except for short sequences that might have relatively high E-values.

receptor kinase 2 (GRK2) as described by Dzimiri *et al.* (2004) and evaluated with the BioRad Beacon designer 3 software. The quality of the RGS4(2) primer pair was rated as "good", while RGS4(1), designed by the Beacon designer software, was rated as the "best" primer pair. The same results was were found for RGS2(2) (see appendix B).

4.1.4 *Temperature gradient for the designed primer oligos of the target genes*

The amplification curves of the primers and cDNA, each at a gradient of temperatures, can be seen in Figure 4-5A, with the corresponding melt curves presented in Figure 4-5B. As the temperature gradients for all primers were tested on one 96-well plate, the results were obtained in one figure, which may seem clustered. Therefore the separate curves can be seen in Appendix B (Figure B-1 to B-12).

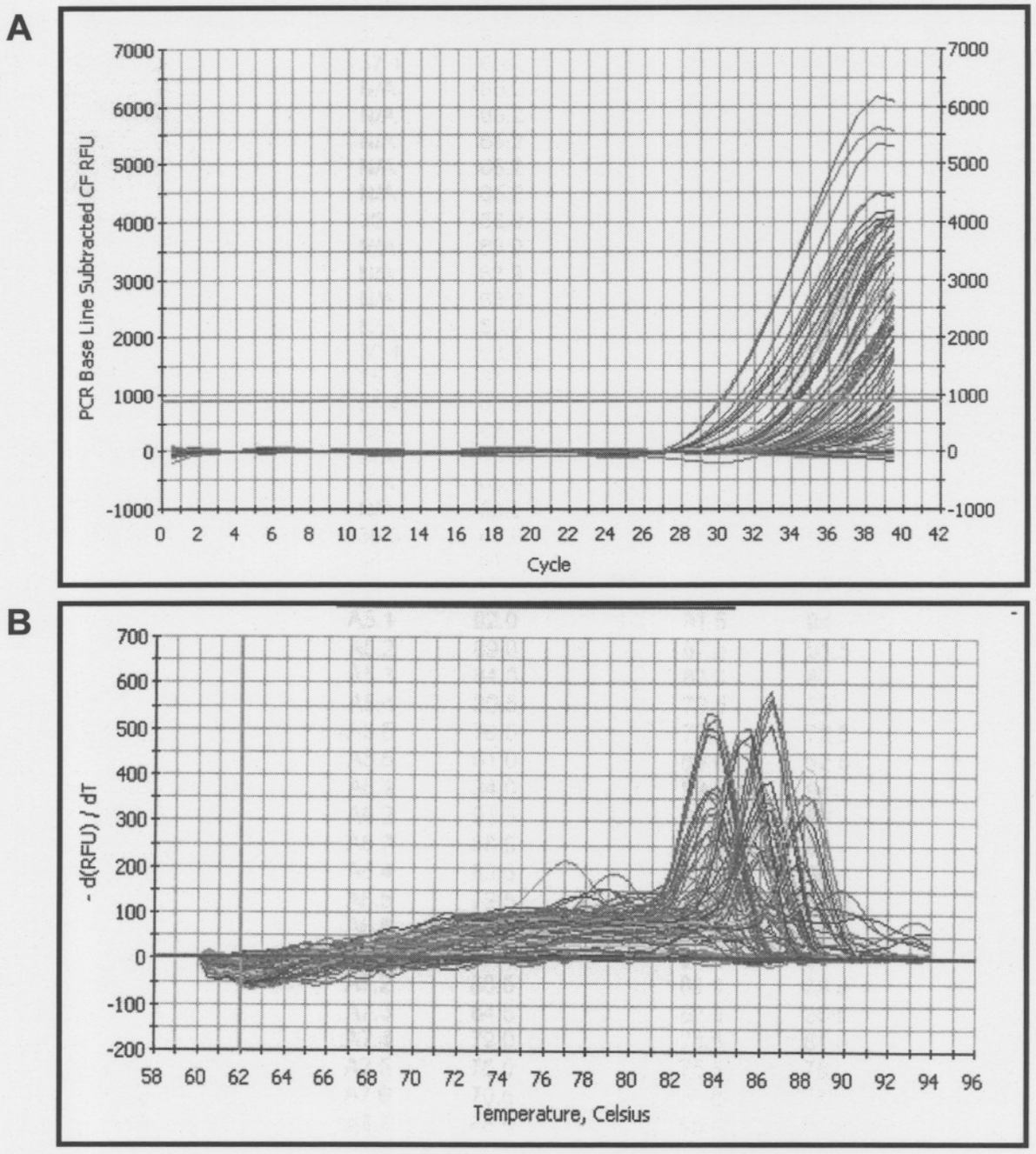


Figure 4-5: *(A) PCR amplification curve of the cDNA with the oligos of the various target genes after a temperature gradient was performed. (B) Melt curve of the amplification product with the oligos of the different target genes after a temperature gradient was performed.*

One peak for a primer pair on a particular melt curve indicates specific primer annealing and no secondary structure or primer dimers formation. As shown in Figure 4-5B the specificity

ranging from non-specific[8] to specific[9] primer annealing. Some of the primers annealed non-specifically even at high temperatures and were discarded from the study.

A temperature gradient was performed, as described in § 3.5.7, in order to determine the optimal annealing temperature for each primer pair. The temperature range was chosen from 55°C[10] to 66°C. The results are shown in Table 4-5.

---

[8] At low temperatures the annealing occurs non-specifically and is identified by two or more melt peaks where more than one amplification product occurs.

[9] Specific annealing occurs at higher annealing temperatures and is identified by only one melt peak (one amplification product).

[10] ± 2°C lower than the lowest theoretical melting temperature (T_m), as predicted by BioRad Beacon designer 3 software.

Table 4-6: *The C_T-values as obtained from Annealing Temperature Gradient experiments for the designed oligos of the target genes*

Annealing Temperature (°C)	PKCγ C_T-value	RGS2(1) C_T-value	RGS4(1) C_T-value	BDNF C_T-value	PLCβ1 C_T-value	GSK3β C_T-value	PKCα C_T-value	PKCε C_T-value	CREB C_T-value	RGS2(2) C_T-value	RGS4(2) C_T-value	GRK2 C_T-value
66.0	36.6	-	-	-	-	-	-	-	-	-	-	-
65.2	37.6	-	-	-	-	-	37.1	-	-	-	-	-
63.9	36.0	-	-	-	-	36.4	35.3	35.4	-	-	-	-
61.8	35.3	36.5	-	-	-	-	33.9	34.8	35.8	-	37.1	-
59.0	36.3	35.5	-	34.8	36.6	36.3	33.3	38.2	32.0	-	35.0	-
57.2	36.0	37.9	-	32.7	34.2	36.4	32.5	35.3	31.0	-	34.2	38.0
55.8	36.5	36.8	37.9	32.5	33.4	34.3	31.4	33.5	30.1	-	34.0	-
55.0	-	36.5	-	31.7	34.3	35.0	31.8	35.0	30.2	36.6	34.1	-

The C_T-value is representative of the cycle at which the amplified product reaches the threshold fluorescence. Table 4-6 shows the C_T-values of the amplification products at the indicated temperatures. The highest temperature where amplification occurred is the optimal annealing temperature for that specific primer pair.

4.1.5 Selection of reverence genes for quantitative real-time RT-PCR

According to the procedure (as described in § 3.5.8) appropriate internal reference (housekeeping) genes were selected. The following table shows the primer sequences of the selected reference genes, from which the best under the experimental conditions implemented, were to be identified.

Table 4-7: *Oligo sequences of the internal reverence genes*

Oligo	Forward Primer sequence	Reverse Primer sequence
ACTB[11]	CTG GAA CGG TGA AGG TGA CA	AAG GGA CTT CCT GTA ACA ATG CA
HPRT1[12]	TGA CAC TGG CAA AAC AAT GCA	GGT CCT TTT CAC CAG CAA GCT
HMBS[13]	GGC AAT GCG GCT GCA A	GGG TAC CCA CGC GAA TCA C
SDHA[14]	TGG GAA CAA GAG GGC ATC TG	CCA CCA CTG CAT CAA ATT CAT G
UBC[15]	ATT TGG GTC GCG GTT CTT G	TGC CTT GAC ATT CTC GAT GGT

These primer sequences were obtained from the Real Time PCR Primer and Probe Database (all submitted by J. O. Vandesompele). In a study done by Vandesompele (2002), several reference genes, the selected ones for this study included, were evaluated in terms of stability and correct normalization. Therefore the primer sequences chosen were

---

[11] Beta actin

[12] Hypoxanthine phosphoribosyl-transferase I

[13] Hydroxymethyl-bilane synthase

[14] Succinate dehydrogenase complex, subunit A

[15] Ubiquitin C

expected to be reliable. After a real time RT-PCR run (as shown in Figure 4-6) the claimed reliability was confirmed.

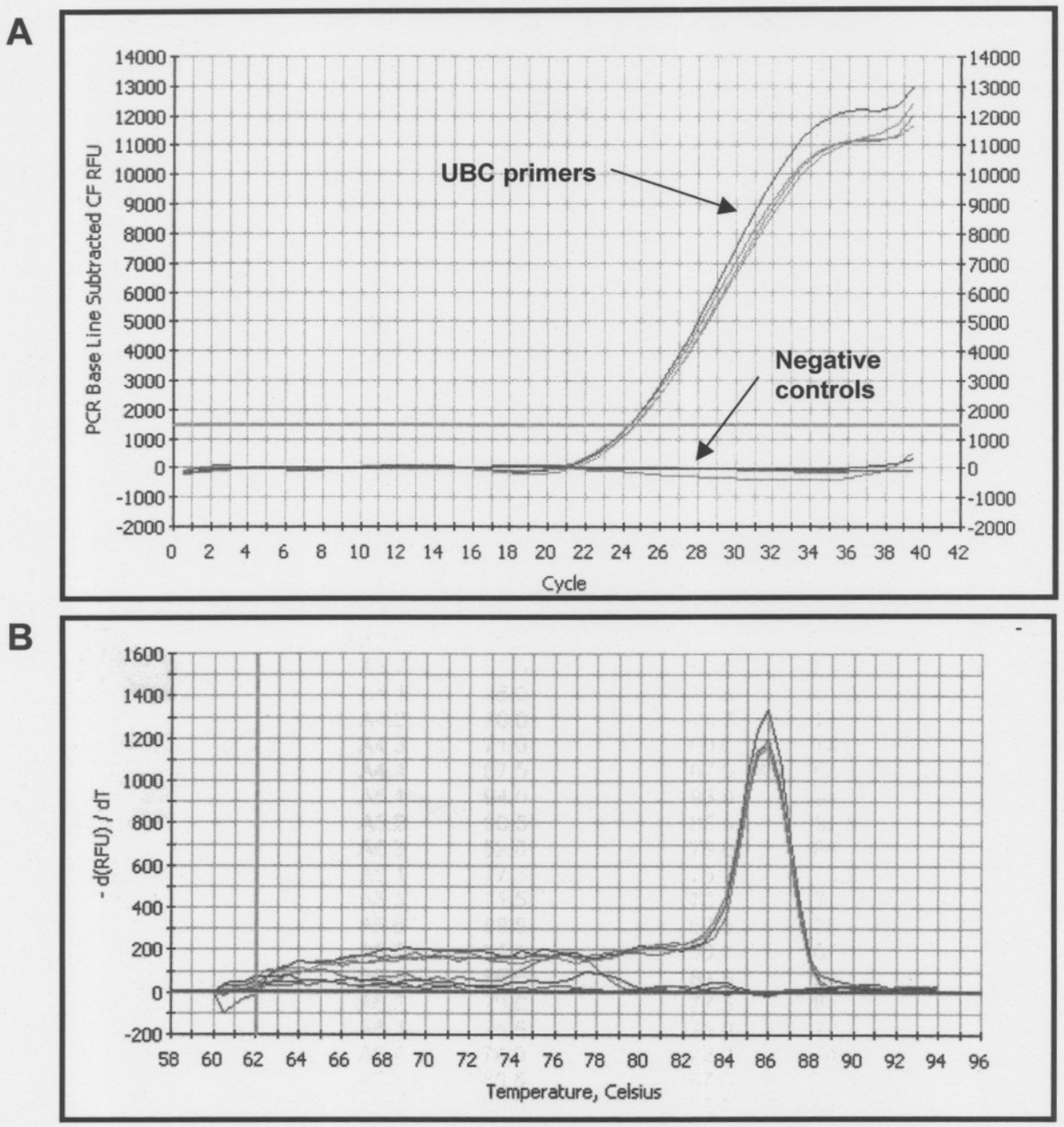


Figure 4-6: *(A) PCR amplification curve of the cDNA from UBC at experimental conditions as described in § 3.5.3. (B) Melt curve of UBC*

The amplification of the UBC reference gene occurred at a threshold cycle (C_T) value of 24. This indicates that an appropriate cDNA concentration was used (between 10-30 cycles – see § 3.5.1.6). Several negative controls (samples without cDNA) were added to the run where no amplification occurred, which confirms that no contamination took place. When

evaluating the melting curve (one peak) it is clear that the primer pair binds specifically to the gene and that no primer dimers or secondary structures are present.

The UBC, HPRT1 and SDHA primer pairs was considered a specifically designed primer suitable for normalization of mRNA quantification.

4.1.6 Real-time RT-PCR Results

In Figure 4-7 the real-time RT-PCR results of PLCβ1 and GSK3β after pre-treatment of the various drugs are shown. The individual amplification and melting curve graphs for the individual target and reference genes are given in Appendix B (Figure B-13 to B-20).

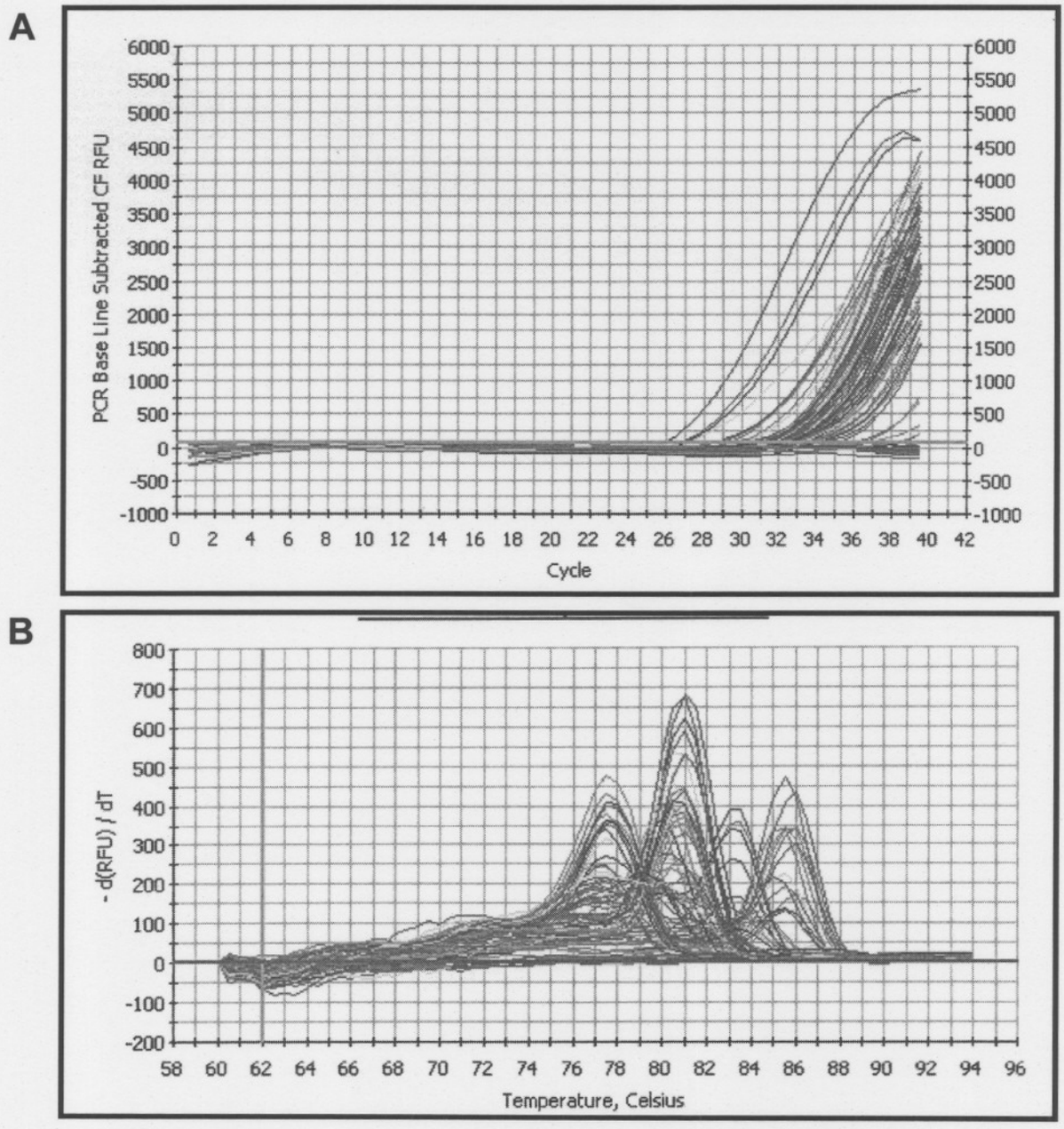


Figure 4-7: *(A) PCR amplification curve of the cDNA for the target genes PLCβ1, GSK3β and reference genes after pre-treatment with the various drugs at experimental conditions as described in §3.5.3. (B) Melt curve of the amplification product from the target genes PLCβ1, GSK3β and the internal reference genes.*

The real-time RT-PCR experiments were performed according to the experimental conditions and procedure described in § 3.5.3. Table 4-8 gives the results as obtained from the quantitative real-time RT-PCR run.

Table 4-8: *Real-time RT-PCR C_T-values for the target genes PLCβ1, GSK3β and the reference genes HPRT1 and SDHA*

Pre-treatment	*n*	*C_T-value (PLCβ1) primers*	*C_T-value (GSK3β primers)*	*C_T-value (HPRT1 primers)*	*C_T-value (SDHA primers)*
Control	1	30.4	26.8	28.6	26.0
	2	34.0	28.8	29.7	27.0
mIns	1	35.3	32.1	31.2	31.2
	2	31.3	28.8	31.7	29.9
FLX	1	N/A	33.0	31.5	32.6
	2	35.9	31.8	31.1	33.4
IMI	1	N/A	31.4	29.4	31.9
	2	38.6	33.5	31.5	33.4
SIL	1	N/A	32.5	32.0	31.1
	2	N/A	32.8	31.7	32.7
LIT	1	N/A	34.2	31.3	31.7
	2	38.3	32.6	32.0	33.6
MIns + FLX	1	N/A	34.1	31.2	32.8
	2	36.8	31.0	31.5	32.9
mIns + SIL	1	N/A	32.5	31.9	33.4
	2	N/A	32.5	31.6	33.6

The quantitative real-time RT-PCR for PLCβ1 after the indicated drug pre-treatments is shown in Figure 4-8:

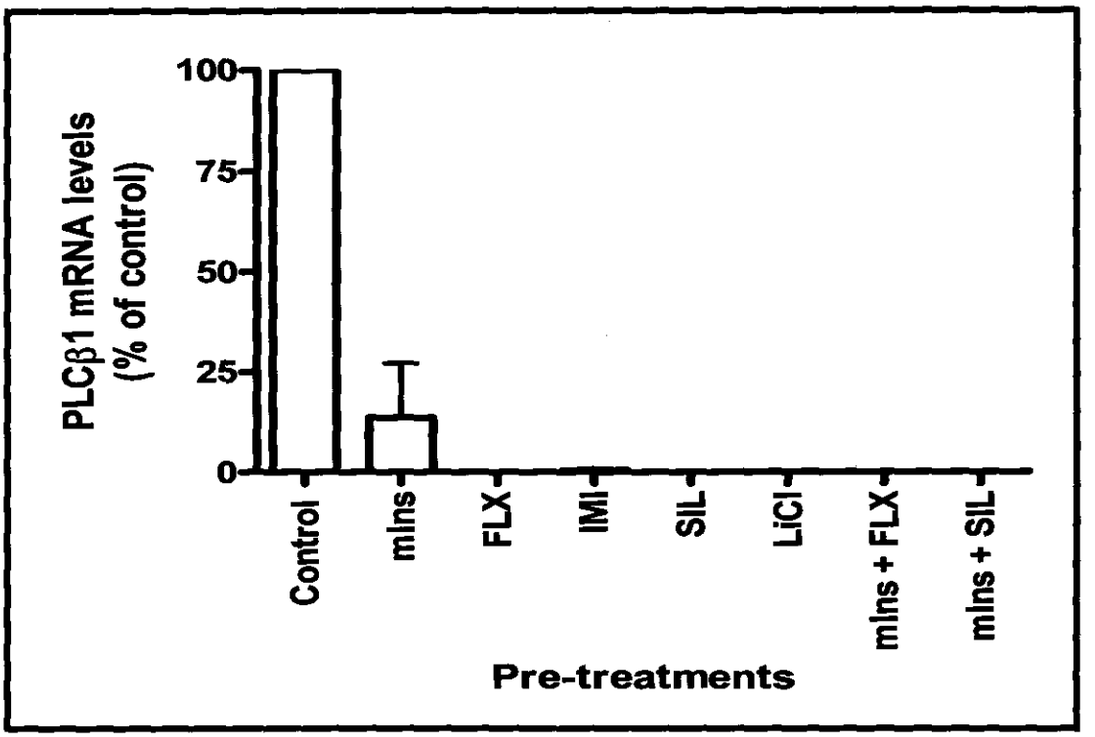


Figure 4-8: *Real-time RT-PCR results of PLCβ1 before and after 24-hour pre-treatments with the indicated drugs normalized with the SDHA reference gene. Bar chart of the relative concentrations of the mRNA for PLCβ1 expression. All data are averages of single observations from two independent experiments, expressed as percentage of control ± standard error of the mean (SEM).*

It can be seen in Figure 4-8 that all drug pre-treatments decreased the PLCβ1 concentration in human neuroblastoma cells. This correlates well with the decreased PLCβ1 concentration found in the membrane fraction of the Western blot results discussed in § 4.1.2.1. However, no increase in the concentration of PLCβ1 after the LiCl pre-treatment can be seen.. The only significant differences between the results from Western blot analyses and the Real-time RT-PCR results include firstly that the Western blot analyses show partial reduction in protein concentration after drug pre-treatments, whereas the Real-time RT-PCR results show a virtually complete inhibition of the mRNA for the expression of these proteins. Secondly, the PLCβ1 protein levels are not reduced after 24-hour pre-treatment with LiCl, whereas the Real-time RT-PCR results suggest that the mRNA levels are already suppressed. This may be due to the time delay between transcriptional and translational events.

The quantitative real-time RT-PCR for PLCβ1 after the indicated drug pre-treatments is shown in Figure 4-8:

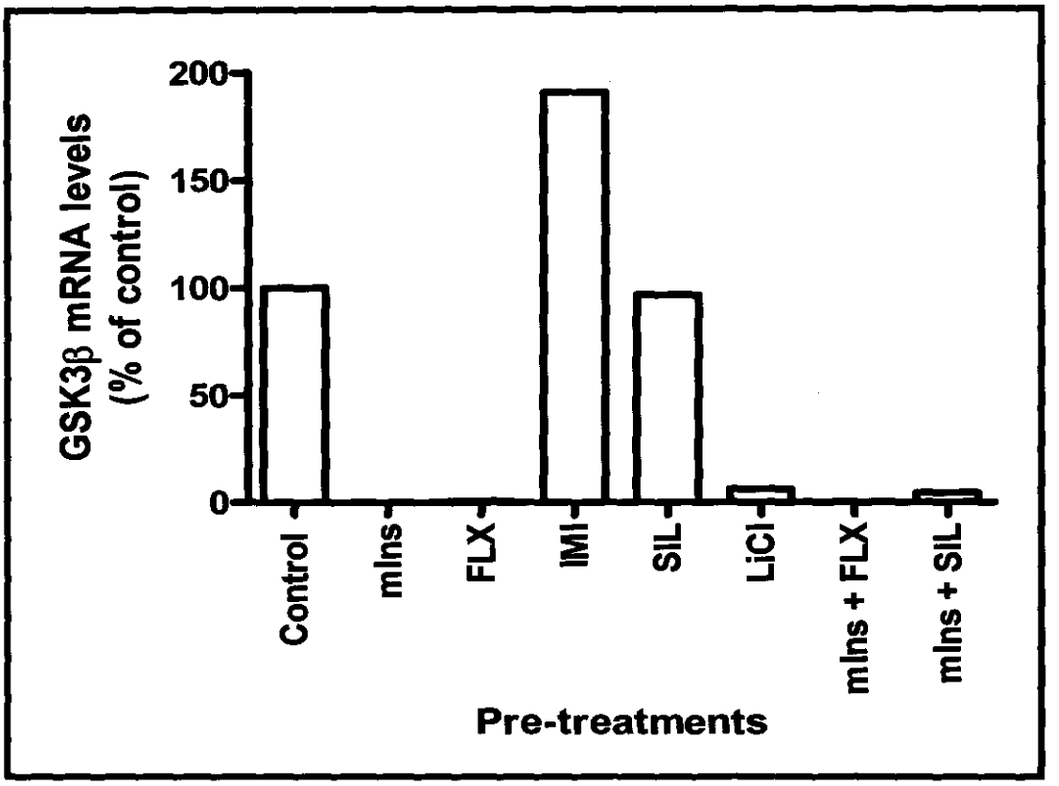


Figure 4-9: *Real-time RT-PCR results of GSK3β before and after 24-hour pre-treatments with the indicated drugs normalized with the SDHA reference gene. Bar chart of the relative concentrations of GSK3β. The data of only a single observation[16] was expressed as percentage of control.*

It can be seen in Figure 4-9 that all drug pre-treatments (except for MI and SIL pre-treatments) decreased the concentration of GSK3β in human neuroblastoma cells. This increase of GSK3β after IMI pre-treatment is not reliable, since the data was obtained from only one experiment[17].

Table 4-8 gives the real-time RT-PCR C_T-values of the cDNA amplification from the target genes, PLCβ1 and GSK3β, and the reference genes, HPRT1 and SDHA, after the indicated drug pre-treatments.

---

[16]Inter-experimental variation occurred (unrealistic values were obtained) and the data were discarded

[17] The experiment needs to be repeated and the results replicated, which was not possible within the time frame and constraints of the current M-Sc study.

The C_T-values (as shown in Table 4-8) were used to calculate relative concentrations for the target genes, PLCβ1 and GSK3β, after the indicated pre-treatments. The C_T-values for the SDHA reference gene[18] were used to calculate relative concentration and to normalize the values for the target genes as described in § 3.6.1 and showed in Figure 4-8 and in Figure 4-9

4.2 Synopsis

In this current study the mRNA levels and protein expression of PLCβ1 and GSK3β before and after various drug pre-treatments were investigated. Results suggest that antidepressant action may include downregulation of PLCβ1 expression. PLCβ1 mRNA levels, but not its protein expression levels, were found to be significantly decreased after lithium pre-treatment. This may suggest the involvement of posttranscriptional modification (or delayed translational effects) of PLCβ1 after lithium treatment.

[18] The melting curves of the amplification products for the SDHA reference gene (Appendix B, Figure B-20) was without secondary products or primer dimers and was therefore used for normalization.

Chapter 5 Summary and Conclusions

5.1 Summary

This study aimed to investigate the possible modulating effect of 24-hour pre-treatments of human neuroblastoma cells with *m*Ins, in comparison to prototype antidepressants and other selected drugs on the mRNA levels of phospholipase C β1 (PLCβ1) and another enzyme implicated in antidepressant action, Glycogen synthase kinase 3β (GSK3β) and the protein expression of PLCβ1 and GSK3β The following experiments were conducted and results obtained:

Western blot assays were successfully optimized and the protein expression of PLCβ1 and GSK3β in the cytosol and membrane fractions were determined. The cytosolic PLCβ1 was unaltered after the pre-treatments with the indicated drugs. The concentration of PLCβ1 in the membrane fraction was statistically significantly decreased after pre-treatment with imipramine (IMI), sildenafil (SIL) and *myo*-inositol (*m*Ins) in combination with FLX and SIL respectively. The concentration of GSK3β in the cytosol and membrane fractions did not reach statistical significance.

Real-time RT-PCR was optimized and the mRNA expression levels were quantified after pre-treatment with the various drug treatments. The mRNA expression was consistent with the protein expression, except for the increased mRNA levels of PLCβ1 after lithium pre-treatment. The mRNA levels of GSK3β remained unaltered, except for the increase in GSK3β after imipramine treatment. The data on mRNA expression levels for GSK3β, however, are not reliable and it is suggested that the experiment be repeated.

Table 5-1 gives a summary of the effects of the indicated drug treatments on the concentrations of PLCβ1 and GSK3β and the respective mRNAs that encode for their expression:

Table 5-1: *Summary of the study results, showing the modulating effects of mIns and the indicated drugs on the protein expression, and mRNA levels for the expression of PLCβ1 and GSK3β. Abbreviations: mIns = myo-inositol; FLX = fluoxetine; IMI = imipramine; SIL = sildenafil; LiCl = lithium chloride*

Drug	PLCβ1			GSK3β		
	Protein Expression		mRNA	Protein Expression		mRNA
	Cytosol	Membrane		Cytosol	Membrane	
mIns	↔	↔	↔	↔	↔	↔
FLX	↔	↔	↓	↔	↔	↔
IMI	↔	↓	↓	↔	↔	↑
SIL	↔	↓	↓	↔	↔	↔
LiCl	↔	↔	↓	↔	↔	↔
mIns + FLX	↔	↓	↓	↔	↔	↔
mIns + SIL	↔	↓	↓	↔	↔	↔

5.2 Conclusion

The data from the current study suggest that antidepressant action may include downregulation of PLCβ1 expression, since the concentration of PLCβ1 was decreased after pre-treatment with imipramine or *m*Ins[1] in combination with fluoxetine. In addition, the phosphodiesterase type 5 (PDE5) inhibitor, sildenafil, alone or in combination with *m*Ins, also decreases the concentration of PLCβ1. These results are consistent with results from a recent study done by Dwivedi *et al.* (2002), who found that all the antidepressants and anxiolytics that cause a decrease in PI-PLC activity also decreased the protein levels of PLCβ1, indicating the role of this specific isozyme in antidepressant and anxiolytic action. Brink *et al.* (2004) found that *m*Ins, FLX and IMI pre-treatments of human neuroblastoma cells (under similar experimental conditions to the current study) reduce muscarinic acetylcholine receptor (mAChR) function and increase 5-HT_{2A} receptor function. This inhibitory effect of these drugs pre-treatments on PLCβ1 could possibly explain the reduction in mAChR function.

---

[1] See § 2.1.2 for a discussion of the antidepressant properties of chronic treatment with high oral doses of *m*Ins.

*m*Ins and fluoxetine pre-treatments also decrease serotonin 5-HT_{2A} receptor (5-HT_{2A}-R) function in human neuroblastoma cells (Brink *et al.*, 2004). Brink *et al.*, (2004) showed that this phenomenon may be explained by an inhibitory effect on 5-HT_{2A}-R signalling at the receptor-G_q protein level, as GTPγS binding to G_q proteins is inhibited by the drug pre-treatments. Since 5-HT_{2A}-Rs also activate PLCβ1, the results from the current study may provide an additional explanation for this observation of reduced 5-HT_{2A}-R function by the drug pre-treatments.

Since PLCβ1 is activated by several G protein-coupled receptors (GPCRs), its downregulation may affect several cellular signalling mechanisms and it still needs to be established whether the currently observed effect is general or confined to, for example, neuronal cells or cells of specific tissue. In addition its relevance in the *in vivo* situation with chronic drug treatment also needs to be confirmed.

In the current study PLCβ1 mRNA levels, but not its protein expression levels, were found to be significantly decreased after lithium pre-treatment. This may suggest the involvement of posttranscriptional modification (or delayed translational effects) of PLCβ1 after lithium treatment. Interestingly, lithium has been shown to inhibit the gene expression or protein expression of several enzymes and GPCR signalling systems in the PI signal-transduction pathway, including inositol monophosphatase (IMPase), 3'(2')-phosphoadenosine 5'-phosphate (PAP) and inositol polyphosphate 1-phosphate (IPPase), as discussed below.

Lithium has been shown to reduce the synthesis of PIs, which are necessary for the PI signalling pathway (Ackenheil, 2001). IMPase is a key enzyme in brain signal transduction and is inhibited by lithium at therapeutic concentrations. Lithium also inhibits IPPase. Lithium decreases the binding of GTP to the G-protein α-subunit (Ackenheil, 2001), but does not change the density of GPCRs after chronic therapy (Gould *et al.*, 2002). Many transcriptional and posttranscriptional events regulate the expression and function of G-proteins, resulting in a virtually unlimited number of possibilities whereby G-protein activity can be modulated in cells (Manji *et al.*, 1996). IMPase is the final inositolpolyphosphate phosphatase prior to conversion to *m*Ins (Freeman *et al.*, 2002). It has been argued, however, that a lithium-induced depletion of *m*Ins may be limited to selected brain areas or even cells that may be particularly vulnerable to this effect, because of restricted *m*Ins supply and/or increased activity of the PI second messenger system (Belmaker *et al.*, 1998). However although chronic lithium administration may affect receptor mediated PI signalling, these effects are unlikely to be simply due to *m*Ins depletion in the CNS. On the other hand, Parthasarathy *et al.* (2003) found that IMPase activity increases substantially in various brain

regions after chronic lithium treatment. They concluded that this finding was due to an adaptation response to chronic lithium treatment and may involve direct or indirect stimulation of the gene or turnover of the enzyme. Shimon *et al.* (1997) also found that brain inositol is reduced post-mortem, although lithium inhibits IMPase. Additionally, in some tissues, $G\alpha_s$ protein couples to Ca^{2+} channels to increase intracellular Ca^{2+} levels. Both increased PI signalling and increased levels of G_α subunits in bipolar patients lead to higher cytosolic Ca^{2+} levels (Ackenheil, 2001). PAP is a recently described lithium inhibitable enzyme. Lithium inhibition of PAP phosphatase may lead to altered gene expression or altered PI second messenger function (Agam *et al.*, 2003).

Lithium has also been shown to inhibit GSK3β, (Meijer *et al.*, 2004) a crucial kinase that functions as an intermediary step in numerous intracellular signalling pathways (Gould *et al.*, 2002). The therapeutic effects of lithium are possibly mediated via the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) signal transduction systems (Ackenheil, 2001). GSK3β lies downstream from the PI3K pathway. In the current study, the mRNA levels and the protein expression of GSK3β remained unaltered after lithium pre-treatment of human neuroblastoma cells. One explanation for this may involve the lithium pre-treatment time of 24 hours, which may have been too short to observe the inhibitory effect of lithium on GSK3β expression (Nonaka *et al.*, 1998). Nonaka *et al.* (1998) found that high concentrations of lithium pre-treatment led not only to loss of the neuroprotective effects but also to a neurotoxic effect. Therefore, a longer pre-treatment with lower concentrations of lithium may have yielded different results.

5.3 Recommendations

Statistical analyses of data from Western blots for PLCβ1 showed no statistically significant differences from control after *m*Ins or FLX pre-treatments, while quantitative PCR suggested a clear reduction in PLCβ1 mRNA. A limited number of repeats of experiments were performed due to constraints during the study. The quality of the data may improved if the experiments are repeated.

Due to the quantitative sensitivity and specificity of the method, future studies could include the investigation of the expression levels of PLCβ1 and GSK3β by using the confocal microscopy to confirm the results found in this study. This method may also reveal any changes in the cellular location of these enzymes.

In addition, the expression levels of PLCβ1 and GSK3β in different brain regions (e.g. the hippocampus, amygdala and frontal cortex) before and after the indicated chronic drug

treatments may be investigated in animal models, to confirm the *in vivo* relevance of the findings of the current study.

In addition, the study may be expanded to include modulatory effects of drug pre-treatments on other enzymes and signalling proteins mentioned in this study, including brain derived neurotropic factor (BDNF), cAMP response element binding protein type 1 (CREB1), regulators of G protein-coupled receptor signalling (RGSs - RGS2 and RGS4), protein kinase C (PKC - PKCα, PKCγ and PKCε) and G protein receptor kinase-2 (GRK2).

References

ACKENHEIL, M. 2001. Neurotransmitters and signal transduction processes in bipolar affective disorders: a synopsis. Journal of Affective Disorders, 62:101-111.

AGAM, G., SHATIEL, G., KOZLOVSKY, N., SHIMON, H., BELMAKER, R.H. 2003. Lithium inhibitable enzymes in postmortem brain of bipolar patients. Journal of Psyciatric Research, 37:433-442.

AMERICAN TYPE CULTURE COLLECTION ATCC. 2004. SH-SY5Y human neuroblastoma cells. [Web:] http://www.atcc.org/SearchCatalogs/Search.cfm [Date of access: 17 Dec. 2004].

ANDERSCH, S., HETTA, J. 2003. A 15-year follow-up study of patients with panic disorder. European Psychiatry, 18:401-408.

AVISSAR, S., SCHREIBER, G. 2002. Toward molecular diagnostics of mood disorders on psychiatry. Trends in molecular medicine, 8(6):294-300.

BALDESSARINI, R.J. 1996. Drugs and the treatment of psychiatric disorders depression and mania. (In Hardman, J.G. & Limbird, L.E. *eds.*, Goodman and Gilman: the pharmacological basis of therapeutics, 9th edition, New York : McGraw-Hill. p 431-459.)

BARNES, N.M., SHARP, T. 1999. A review of central 5-HT receptors and their function. Neuro Pharmacology, 38:1083-1152.

BARRY, F.A., GRAHAM, G.J., FRY, M.J., GIBBINS, J.M. 2003. Regulation of glycogen synthase kinase 3 in human platelets: a possible role in platelet function? Federation of European biochemical societies letters, 553:173-178.

BECKER, R.W.,TYOBEKA, E.M. 1998. Second messenger responses during lithium enhanced proliferation of HL-60 promyelocytic leukemia cells: cAMP and the inositol phosphates. (In Becker, R.W., Lucas, K.C., Gallicchio, V.S., The biological and clinical actions of lithium: new perspectives, p55-66.)

BELMAKER, R.H., BERSUDSKY, Y., BENJAMIN, J., AGAM, G., LEVINE, J., KOFMAN, O. 1995. Manupulation of inositol-linked second messenger system as a therapeutic strategy in psychiatry. (In GESSA, G.L., FRATTA, W., PANI, L., SERRA, G., *eds.* New York : Raven press. p. 67-84.)

BELMAKER, R.H., AGAM, G., VAN CALKER, D., RICHARDS, M.H., KOFMAN, O. 1998. Behavioral reversal of lithium effects by four inositol isomers correlates perfectly with biochemical effects in the PI cycle: depletion by chronic lithium of brain inositol is specific to hypothalamus, and inositol levels may be abnormal in postmortem brain from bipolar patients. Neoropsychopharmacology, 19(3):220-232.

BENEDETTI, F., SERRETTI, A., COLOMBO, C., LORENZI, C., TUBAZIO, V., SMERALDI, E. 2004. A glycogen synthase kinase 3-β promoter gene single nucleotide polymorphism is associated with age at onset and response to total sleep deprivation in bipolar depression. Neuroscience letters, 368:123-126.

BLIER, P., DE MONTIGNY, C. 1999. Serotonin and drug-induced therapeutic responses in major depression, obsessive-compulsive and panic disorder. Neuropsychopharmacology, 21(2):91s-98s.

BRAILOIU, E., MIYAMOTO, D., DUN, N. J. 2003. Inositol derivatives modulate spontaneous transmitter release at the frog neuromuscular junction. Neuropharmacology, 45:691-701.

BRUNELLO, N., MENDLEWICZ, J., KASPER, S., LEONARD, B., MONTGOMERY, S., NELSON, J.G., PAYKEL, E., VERSIANI, M., RACAGNI, G. 2002. The role of noradrenaline and selective noradrenaline reuptake inhibition in depression. European neuropsychopharmacology, 12:461-475.

CASTRÉN, E. 2004. Neurotrophic effects of antidepressant drugs. Current Opinion in Pharmacology, 4:58-64

CHEN, B., DOWLATSHAHI, D., MACQUEEN, G. M., WANG, J. F., YOUNG, L. T. 2001. Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. Biological Psychiatry, 50:260-265.

COCCO, L., CAPITANI, S., BARNABEL, O., GILMOUR, R.S., RHEE, S.G., MANZOLI, F.A. 1999. Inositides in the nucleas: further developments on phospholipase Cβ1 signalling during erythroid differentiation and IGF-I induced mitogenesis. Advances in Enzyme Regulation, 39:287-297.

COCCO, L., MARTELLI, A.M., VITALE, M., FALCONI, M., BARNABEI, O., GILMOUR, R.S., MANZOLI, F.A. 2002. Inositides in the nucleus: regulation of nuclear PI-PLCβ1. Advances in Enzyme Regulation, 42:181-193.

DAZZI, L., VACCA, G., LADU., S., PISU M.G., SERRA, M., BIGGIO, G. 2001. Long-term treatment with antidepressant drugs reduces the sensitivity of cortical cholinergic neurons to the activating actions of stress and the anxiogenic drug FG 7142. Neuropharmacology, 41:229-237.

DE KOCK, S.E. 2003. The role of myo-inositol in G-protein coupled receptor-mediated sub-cellular transduction mechanisms in neuronal cell lines. Potchefstroom : PU for CHE. (Dissertation – M.Sc.) 97 p.

DJORDJEVIC, S., DRISCOLL, P.C. 2002. Structural insight into substrate specificity and regulatory mechanisms of phosphoinositide 3-kinase. Trends in biochemical sciences, 27(8):426-432.

DUMAN, R. S. 2002. Pathophysiology of depression: the concept of synaptic plasticity. European Psychiatry, 17(suppl 3):306-310

DUMAN, R.S., MALBERG, J., THOME, J. 1999. Neural plasticity to stress and antidepressant treatment. Biological Psychiatry, 46:1181-1191.

DWIVEDI, Y., AGRAWAL, A.K., RIZAVI, H.S., PANDEY, G.N. 2002. Antidepressants reduce phosphoinositide-specific phospholipase C (PI-PLC) activity and the mRNA and protein expression of selective PLC β_1 isozyme in rat brain. Neuropharmacology, 43:1269-1279.

DZIMIRI, N., MUIYA, P., ANDRES, E., AL-HALEES, Z. 2004. Differential functional expression of human myocardial G protein receptor kinases in left ventriclular cardiac diseases. European journal of pharmacology, 489:167-177.

EINAT, H., BELMAKER, R.H. 2001. The effects of inositol treatment in animal models of psychiatric disorders. Journal of affective disorders, 62:113-121.

FISHER, S.K., NOVAK, J.E., AGRANOFF, B.W. 2002. Inositol and higher inositol phosphates in neuronal tissues: homeostasis, metabolism and functional significance. Journal of neurochemistry, 82:1-19.

FREEMAN, M.P., FREEMAN, S.A., MCELROY, S.L. 2002. The comorbidity of bipolar and anxiety disorders: prevalence, psychobiology, and treatment issues. Journal of Affective Disorders, 68:1-23.

FRUMAN, D.A., MEYERS, R.E., CANTLEY, L.C. 1998. Phosphoinositide kinases. Annual Reviews of Biochemistry, 67:481-507.

GERSON, E.S. 1995. Recent developments in genetics of bipolar illness. (In GESSA, G.L., FRATTA, W., PANI, L., SERRA, G., *eds*. New York : Raven press. p. 85-98.)

GOODMAN, W.K., BYERLY, M., MURPHY, T. 1995. Treatment of severe OCD. European Neuropsychopharmacology, 5 (3):163-164.

GOODMAN, W.K., WARD, H., KABLINGER, A., MURPHY, T. 1997. Fluvoxamine in the treatment of obsessive-compulsive disorder and related conditions. Journal of clinical psychiatry, 58:32-49.

GOULD, T.D., CHEN, G., MANJI, H.K. 2002. Mood stabilizer psychopharmacology. Clinical Neuroscience Research, 2:193-212.

GREENBERG, B.D., BENJAMIN, J., MARTIN, J.D., KEULER, D., HUANG, S.J., ALTEMUS, M., MURPHY, D.L. 1998. Delayed obsessive-compulsive disorder symptom exacerbation after a single dose of a serotonin antagonist in fluoxetine-treated but not untreated patients. Psychopharmacol. (Berl.), 40:434-444.

GURGUIS, G.N.M., ANTAI-OTONG, D., VO, S.P., BLAKELEY, J.E., ORSULAK, P.J., PETTY, F., RUSH, A.J. 1999. Adrenergic receptor function in panic disorder. Neuropsychopharmacology, 20(2):162-176.

HARVEY, B.H. 1997. The neurobiology and pharmacology of depression a comparative overview of serotonin selective antidepressants. SAMJ Psychiatry, 87(4):540-552.

HARVEY, B.H., BRINK, C.B., SEEDAT, S., STEIN, D.J. 2002. Defining the neuromolecular action of myo-inositol application to obsessive-compulsive disorder. Progress in neuro-psychopharmacology & biological psychiatry, 26:21-32.

HARVEY, B.H., SCHEEPERS, A., BRAND, L., STEIN, D.J. 2001. Chronic inositol increases striatal D2 receptors but does not modify dexamphetamine-induced motor behavior: relevance to obsessive-compulsive disorder. Pharmacology, biochemistry and behavior, 68:245-253.

HEMMINGS, S.J., KINNEAR, C.J., NIEHAUS, D.J.H., MOOLMAN-SMOOK, J.C., LOCHNER, C., KNOWLES, J.A., CORFIELD, V.A., STEIN, D.J. 2003. Investigating the role of dopaminergic and serotonergic candidate genes in obsessive-compulsive disorder. European neuropsychopharmacology, 13:93-98.

HOLSBOER, F. 2001. Prospects for antidepressant drug discovery. Biological psychology, 57:47-65.

HORROBIN, D.F., BENNETT, C.N. 1999. New gene targets related to schizophrenia and other psychiatric disorders: enzymes, binding proteins and transport proteins involved in phospholipid and fatty acid metabolism. Prostaglandins, leukotrienes and essential fatty acids, 60(3):141-167.

JENIKE, M.A. 2001. An update in obsessive-compulsive disorder. Bull menninger Clin., 65:4-25.

JOPE, R.S. 2004. Inhibition of glycogen synthase kinase-3: a potential therapeutic target of lithium. Clinical neuroscience research, p1-9 (Article in Press).

KASPER, S., RESINGER, E. 2001. Panic disorder: the place of benzodiazepines and selective serotonin reuptake inhibitors. European neuropsychopharmacology, 11:307-321.

KIM, M., PARK, S., SHIN, M.S., KWON, J.S. 2002. Neuropsychological profile in patients with obsessive-compulsive disorder over a period of 4-month treatment. Journal of Psychiatric Research, 36:257-265.

LARMINIE, C., MURDOCK, P., WALHIN, J., DUCKWORTH, M., BLUMER, K.J., SCHEIDELER, M.A., GARNIER, M. 2003. Selective expression of regulators of G-protein signaling (RGS) in the human central nervous system. Molecular brain research, 122:24-34.

LESCH, K.P. 2001. Serotonergic gene expression and depression: implications for developing novel antidepressants. Journal of Affective Disorders, 62:57-76.

LEONARD, B.E. 2003. Fundamentals of Psycopharmacology, 3rd edition. West Sussex : Wiley & Sons. 520 p.

LEVINE, J. 1997. Controlled trails of inositol in psychiatry. European neuro-psychopharmacology, 7:147-155.

LYKOURAS, L., ALEVIZOS, B., MICHALOPOULOU, P., RABAVILAS, A. 2002. Obsessive-compulsive symptoms induced by atypical antipsychotics. A review of the reported cases. Progress in neuro-psychopharmacology and biological psychiatry, 27:333-346.

MANJI, H.K., BERSUDSKY, Y., CHEN, G., BELMAKER, R.H., POTTER, W.Z. 1996. Modulation of protein kinase C isozymes and substrates by lithium: the role of myo-inositol. Neuropsychopharmacology, 15(4):370-381.

MANJI, H.K., QUIROZ, J.A., SPORN, J., PAYNE, J.L., DENICOFF, K., GRAY, N.A., ZARATE, C.A., CHARNEY, D.S. 2003. Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. Biological Psychiatry, 53:707-742.

MARCUS, R. & COULSTON, A.M. 1996. Water-soluble vitamins: the vitamin B complex and ascorbic acid. (In Hardman, J.G. & Limbird, L.E. *eds*., Goodman and Gilman: the pharmacological basis of therapeutics, 9th edition, New York : McGraw-Hill. p. 1555-1572.)

MEIJER, L., FLAJOLET, M., GREENGARD, P. 2004. Pharmacological inhibitors of glycogen synthase kinase 3. Trends in Pharmacological Sciences, 25(9):471-480.

MISHRA, J., BHALLA, U.S. 2002. Stimulations of inositol phosphate metabolism and its interaction with $InsP_3$-mediated calcium release. Biophysical Journal, 83:1298-1316.

MOORE, G.J., BEBCHUK, J.M., PARRISH, J.K., FAULK, M.W., ARFKEN, C.L., STRAHL-BEVACQUA, J.,MANJI, H. 1999. Temporal dissociation between lithium-induced changes in frontal lobe myo-inositol and clinical response in manic-depressive illness. American Journal of Psychiatry, 156(12)1902-1908.

NADRI, C., DEAN, B., SCARR, E., AGAM, G. GSK-3 parameters in postmortem frontal cortex and hippocampus of schizophrenic patients. Schizophrenia Research, 71:377-382.

NATIONAL CENTRE FOR BIOTECHNOLOGY INFORMATION (NCBI). 2003. Genbank sequence blast database [Web] (www.ncbi.nlm.nih.gov). [Date of access: Jun. 2003].

NESTLER, E.J., GOULD, E., MANJI, H., BUCAN, M., DUMAN, R.S., GERSHENFELD, H.K., HEN, R., KOESTER, S., LEDERHENDLER, I, MEANEY, M.J., ROBBINS, T., WINSKY, L., ZALCMAN, S. 2002. Preclinical models: status of basic research in depression. Biological Psychiatry, 52:503-528.

NONAKA, S., HOUGH, C.J., CHUANG, D. 1998. Chronic lithium treatment robustly protects neurons in the central nervous system against exitotoxicity by inhibiting N-methyl-D-aspartate receptor-mediated calsium influx. Proc. Natl. Acad. Sci, USA, 95:2642-2647.

OFFERMANNS, S. 2003. G-proteins as signal transducers in transmembrane signaling. Progress in biophysics and molecular biology, 83:101-130.

OKEN, R.J. 2001. Obsessive-compulsive disorder: a neuronal membrane phospholipid hypothesis and concomitant therapeutic strategy. Medical hypotheses, 56(4):413-415.

PANDEY, G.N., DWIVEDI, Y., PANDEY, S.C., TEAS, S.S., CONLEY, R.R., ROBERTS, R.C., TAMMINGA, C.A. 1999. Low phosphoinositide-specific phospholipase C activity and expression of phospholipase C beta1 protein in the prefrontal cortex of teenage suicide subjects. American Journal of Psychiatry, 156:1895-1901.

PANDEY, G.N., DWIVEDI, Y., SRIDHARARAO, J., REN, X., JANICAK, P.G., SHARMA, R. 2002. Protein kinase C and phospholipase C activity and expression of their specific isozymes is decreased and expression of MARCKS is increased in platelets of bipolar but not in unipolar patients. Neuropsychopharmacology, 26(2):216-228.

PARTHASARATHY, L.K., SEELAN, R.S., WILSON, M.A., VANDAL, R.E., PARTHASARATHY, R.N. 2003. Regional changes in rat brain inositol monophosphatase 1 (IMPase 1) activity with chronic lithium treatment. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 27:55-60.

PAULS, D.L., RAYMOND, C.L., STEVENSON, J.M., LECKMAN, J.F. 1991. A family study of Gilles de la Tourette syndrome. American J. Hum. Genet., 48:154-163.

PREECE, P., VIRLEY, D.J., COSTANDI, M., COOMBES, R., MOSS, S.J., MUDGE, A.W., JAZIN, E., CAIRNS, N.J. 2003. Beta-secretase (BACE) and GSK-3 mRNA levels in Alzheimer's disease. Brain res Mol brain res, 116(1/2): 155-158.

RAHMAN, S., NEUMAN, R.S., 1993. Myo-inositol reduces serotonin (5-HT_2) receptor induced homologous and heterologous desensitization. Brain research, 631:349-351.

RAMASUBBU, R., RAVINDRAN, A., LAPIERRE, Y. 2000. Serotonin and dopamine antagonism in obsessive-compulsive disorder: effect of atypical antipsychotic drugs. Pharmacopsychiatry, 33:236-238.

RAMEH, L.E., CANTLEY, L.C. 1999. The role of phosphoinositide 3-kinase lipid products in cell function. The Journal of Biological Chemistry, 274(13):8347-8350.

REAL TIME PCR PRIMER AND PROBE DATABASE. Primer sequence blast database [Web:] (http://medgen.ugent.be/rtprimerdb/). [Date of access: Jun 2003]

RICCIO, A., ANH, S., DAVENPORT, C.M., BLENDY, J.A., GINTY, D.D. 1999. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science, 286:2358-2361.

ROSENBERG, D.R., HANNA, G.L. 2000. Genetic and imaging strategies in obsesseve-compulsive disorder: potential implications for treatment development. Biological Psychiatry, 48:1210-1222.

ROTHENBERGER, A. 1994. Neurobiology and drug treatment of Tourette's syndrome and other tic disorders. European neuropsychopharmacology, 4(3):245-247.

RYDER, J., SU, Y., NI, B. 2004. Akt/GSK3β serine/threonine kinases: evidence for a signalling pathway mediated by familial alzheimer's disease mutations. Cellular signalling, 16:187-200.

SASSON, Y., IANCU, I., FUX, M., TAUB, M., DANNON, P.N., ZOHAR, J. 1999. A double-blind crossover comparison of clomipramine and desipramine in the treatment of panic disorder. European neuropsychopharmacology, 9:191-196.

SCHWARTZ, Z., SHAKED, D., HARDIN, R.R., GRUWELL, S., DEAN, D.D., SYLVIA, V.L., BOYAN, B.D. 2003. $1\alpha,25(OH)_2D_3$ causes a rapid increase in phosphatidylinositol-specific PLC-β activity via phospholipase A_2-dependent production of lysophospholipid. Steroids, 68:423-437.

SHIMON, H., AGAM, G., BELMAKER, R.H., HYDE, T.M., KLEINMAN, J.E. 1997. Reduced frontal cortex inositol levels in postmortem brain of suicide victims and patients with bipolar disorder. American journal of psychiatry, 154:1148-1150.

SHIMON, H., YELANA, S., DAVIDSON, M., HAROUTUNIAN, V., BELMAKER, R.H., AGAM, G. 1998. Inositol levels are decreased in postmortem brain of schizophrenic patients. Biological Psychiatry, 44:428-432.

SOARES, J.C., CHEN, G., DIPPOLD, C.S., WELLS, K.F., FRANK, E., KUPPFER, D.J., MANJI, H.K., MALLINGER, A. G. 2000. Concurrent measures of protein kinase C and

phosphoinositide in lithium treated bipolar patients and healthy individuals: a preliminary study. Psychiatry Res, 95:109-118.

STAGLIANO, K.E.,R., CARCHMAN, E., DEB, S. 1994. Real-time polymerase chain reaction quantitation of relative expression of genes modulated by p53 using SYBR® Green I. (In Deb, S. & Deb, S.P., Methods in molecular biology, vol. 234: p53 protocols, Totowa : Humana Press Inc. p73 –91.

STAHL, S.M. 2002. Essential psychopharmacology: neuroscientific basis and practical applications, 2nd edition. Cambridge : Cambridge university press. 601p.

STEIN, D.J., SPADACCINI, E., HOLLANDER, E. 1995. Meta-analysis of pharmacotherapy trials for obsessive-compulsive disorder. Int. Clin. Psychopharmacol, 10:11-18.

SZECHTMAN, H., SULIS, W., EILAM, D. 1998. Quinpirole induces compulsive checking behavior in rats: a potential animal model of obsessive-compulsive disorder (OCD). Behavioural Neuroscience, 112:1475-1485.

SZECHTMAN, H., CULVER, K., EILAM, D. 1999. Role of dopamine systems in obsessive-compulsive disorder (OCD): implications from a novel psychostimulant-induced animal model. Pol J Pharmacol, 51:55-61.

TAMMINGA, C.A., NEMEROFF, C.B., BLAKELY, R.D., BRADY, L., CARTER, C.S., DAVIS, K.L., DINGLEDINE, R., GORMAN, J.M., GRIGORIADIS, D.E., HENDERSON, D.C., INNIS, R.B., KILLEN, J., LAUGHREN, T.P., MCDONALD, W.M., MURPHY, G.M., JR., PAUL, S.M., RUDORFER, M.V., SAUSVILLE, E., SCHATZBERG, A.F., SCOLNICK, E.M., SUPPES, T. 2002. Developing novel treatments for mood disorders: accelerating discovery, 52:589-609.

TARDITO, D., ZANARDI, R., RACAGNI, G., MANZORI, T., PEREZ, J. 2002. The protein kinase A in platelets from patients with panic disorder. European neuropsychopharmacology, 12:483-487.

THOME, J., SAKAI, N., SHIN, K., STEFFEN, C., ZHANG, Y.J., IMPEY, S. STORM, D., DUMAN, R.S. 2000. cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. The journal of neurosciene, 20(11):4030-4036.

THUMMEL, K.E., SHEN, D,D. 2001. Design and optimization of dosage regimens: Pharmacokenetic data. (In Hardman, J.G. & Limbird, L.E. *eds.*, Goodman & Gilman: the pharmacological basis of therapeutics, 10th edition, New York : McGraw-Hill. p. 1917-2023.)

ULLOA, R., NICOLINI, H., FERNÁNDEZ-GUASTI, A. 2004. Age difference in an animal model of obsessive-compulsive disorder: participation of dopamine: dopamine in an animal model of OCD. Pharmacology, biochemistry and behavior, 78:661-666.

.VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3(7):1-12.

VILJOEN, S.L. 2002. The effects of myo-inositol on mAChR expression and function. Potchefstroom: PU for CHE. (Dissertation – M.Sc.) 100 p.

WACHIRA, S.J.M., MAMPURU, L.J., SHAI, L.J., TYOBEKA, E.M., ABOTSI, E.K. 1998. Analysis of the molecular mechanism of lithium on mammalian cells in culture. (In Becker, R. W., Lucas, K.C., Gallicchio, V.S., The biological and clinical actions of lithium: new perspectives, p.67-82.)

WELKOWITZ, L.A. 2000. Obsessive-compulsive disorder and comorbid anxiety problems in a national anxiety screening sample. Journal of Anxiety Disorders, 14(5):471-482.

WOOD , M.D., REAVILL, C., TRAIL, B., WILSON, A., STEAN, T., KENNETT, G.A., LIGHTOWLER, S., BLACKBURN, T.P., THOMAS, D., GAGER, T.L., RILEY, G., HOLLAND, V., BROMIDGE, S.M., FORBES, I.T., MIDDLEMISS, D.N. 2001. SB-243213; a selective 5-HT2C receptor inverse agonist with improved anxiolytic profile: lack the tolerance and withdrawel anxiety. Neuropharmacology, 41:186-199.

YAMADA, M., HIGUCHI, T. 2002. Functional genomics and depression research beyond the monoamine hypothesis. European Neuropsychopharmacology, 12:235-244.

YUAN, P., GOULD, T.D., GRAY, N.A., BACHMAN, R.F., SCHLOESSER, R.J., LAN, M., J.K., DU, J., MOORE, G.J., MANJI, H.K. 2004. Neurotrophic signaling cascades are major long-term targets for lithium: clinical implications. Clinincal neuroscience research, p1-17 (Article in Press).

List of Figures

List of Tables

Appendix A

A.1. Table of abbreviations

Abbreviation	Description
5-HT	Serotonin
5-HT_1	Serotonin 1
5-HT_{1A}	Serotonin 1A
5-HT_{1B}	Serotonin 1B
5-HT_2	Serotonin 2
5-HT_{2A}	Serotonin 2A
5-HT_{2A}-R	Serotonin 2A receptor
5-HT_{2C}	Serotonin 2C
5-HT_{2C}-R	Serotonin 2C receptor
5-HT_3	Serotonin 3
AC	Adenylyl cyclase
ACTB	Beta actin
Akt	Protein kinase B
AP	Atypical antipsychotics
ATCC	AmericanType Culture Colllection
B2M	Beta-2-microglobulin
BDNF	Brain derived neurotrophic factor
bp	Base pair
C	Cytidine
Ca^{2+}	Calcium
cAMP	Cyclic adenosine mono-phosphate
cDNA	Complementary DNA

CDP-DAG	Cytidine diphosphadiacylgycerol
cGMP	Cyclic guanosine mono-phosphate
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CRE	cAMP response element
CREB	cAMP response element binding protein
CREB1	cAMP response element binding protein 1
CSTC	Cortico-striatal-thalamo-cortical
C_T	Threshold cycle
D_2	Dopamine 2
D_4	Dopamine 4
DAG	Diacylglycerol
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine 5'-triphosphate
DMEM	Dulbecco's Modified Eagles Medium
DNA	DeoxyriboNucleic Acid
DNase	Deoxyribonuclease
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphate
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
dTTP	Thymidine 5'-triphosphate
ECL	Enhanced chemiluminesence
ECT	Electroconvulsive therapy
EDTA	Ethylenediaminetetra acetic acid
EMEM	Minimum Essential Media with Earle's Base
EtBr	Ethidium Bromide
FBS	Fetal bovine serum
FLX	Fluoxetine

G	Guanosine
GABA	Gamma-amino butyric acid
GABA-A	Gamma-amino butyric acid A
GABA-B	Gamma-amino butyric acid B
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
G-protein	Guanosine triphosphate binding protein
GRK2	G-protein receptor kinase 2
GSK	Glycogen synthase kinase
GSK 3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HMBS	Hydroxymethyl-bilane synthase
HPRT1	Hypoxanthine phosphoribosyl-transferase I
IMI	Imipramine
IMPase	Inositol monophosphate phosphatase
IP_2	Inositol 1,4-biphosphate
IP_3	Inositol triphosphate
IP_4	Inositol 1,3,4,5-tetrakisphosphate
IP_5	Inositol 1,3,4,5,6-pentakisphosphate
IP_6	Inositol 1,2,3,4,5,6-hexakisphosphate
IPPase	Inositol polyphosphate 1-phosphate
LiCl	Lithium
l-N_2	Liquid nitrogen
l-NE	l-norepinephrine
mAChR	Muscarinic acetylcholine receptor
MAOA	Monoamine Oxidase A
MAOB	Monoamine Oxidase B
MAOI	Monoamine Oxidase Inhibitors
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase

$MgCl_2$	Magnesium Chloride
*m*Ins	*myo*-inositol
mRNA	Messenger RiboNucleic Acid
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NE	Norepinephrine
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
P57	Pan-neurotrophin receptor
PA	Phosphatidic acid
PAP	3'(2')-phosphoadenosine 5'phosphate
PBS	Phosphate buffered saline
PI	Phosphatidyl inositol
PI-3K	Phosphoinositide 3-kinase
PIP2	Phosphatidyl-inositol 4,5-biphosphate
PI-PLC	Phosphoinositide specific-phospholipase C
PKA	cAMP-dependant protein kinase
PKC	Protein kinase C
PKCα	Protein kinase Cα
PKCγ	Protein kinase Cγ
PKCε	Protein kinase Cε
PLC	Phospholipase C
PLC-β1	Phospholipase C β1
PLCγ	PLC gamma
PMSF	Phenylmethylsulphonyl fluoride
RGS	Regulators of G protein-coupled receptor signalling
RGS2	Regulators of G protein-coupled receptor signalling 2
RGS4	Regulators of G protein-coupled receptor signalling 4
RNA	Ribonucleic acid
RNase	Ribonuclease

RPL13A	Ribosomal protein L13a
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RTPrimerDB	Real Time PCR Primer and Probe Database
SDHA	Succunate dehydrogenase complex, subunit A
SEM	Standard error of the mean
SH-SY5Y	Human neuroblastoma cell line
SIL	Sildenafil
SSRIs	Serotonin selective reuptake inhibitors
TBP	TATA box binding protein
TCAs	Trycyclic antidepressants
TEMED	N,N,N',N-Tettamethylethylenediamine
TF	Nuclear transcription factor
T_m	Melting temperature
TPH	Tryptophan hydroxylase
TRIS	Tris(hydroxymethyl)aminomethane
TRIS-base	Tris(hydroxymethyl)aminomethane base
TRIS-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TrkB	Tyrosine receptor kinase
Tween® 20	polyoxethylene sorbitan monolaurate
UBC	Ubiquitin C
UV	Ultraviolet
Wnt	Wingless type pathway
YWHAZ	Tyrosine 3-monooxegenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
α_1-ARs	α_1-adrenergic receptors
α-ARs	α-adrenergic receptors

Appendix B

B.1 Phospholipase C β1 mRNA Sequence

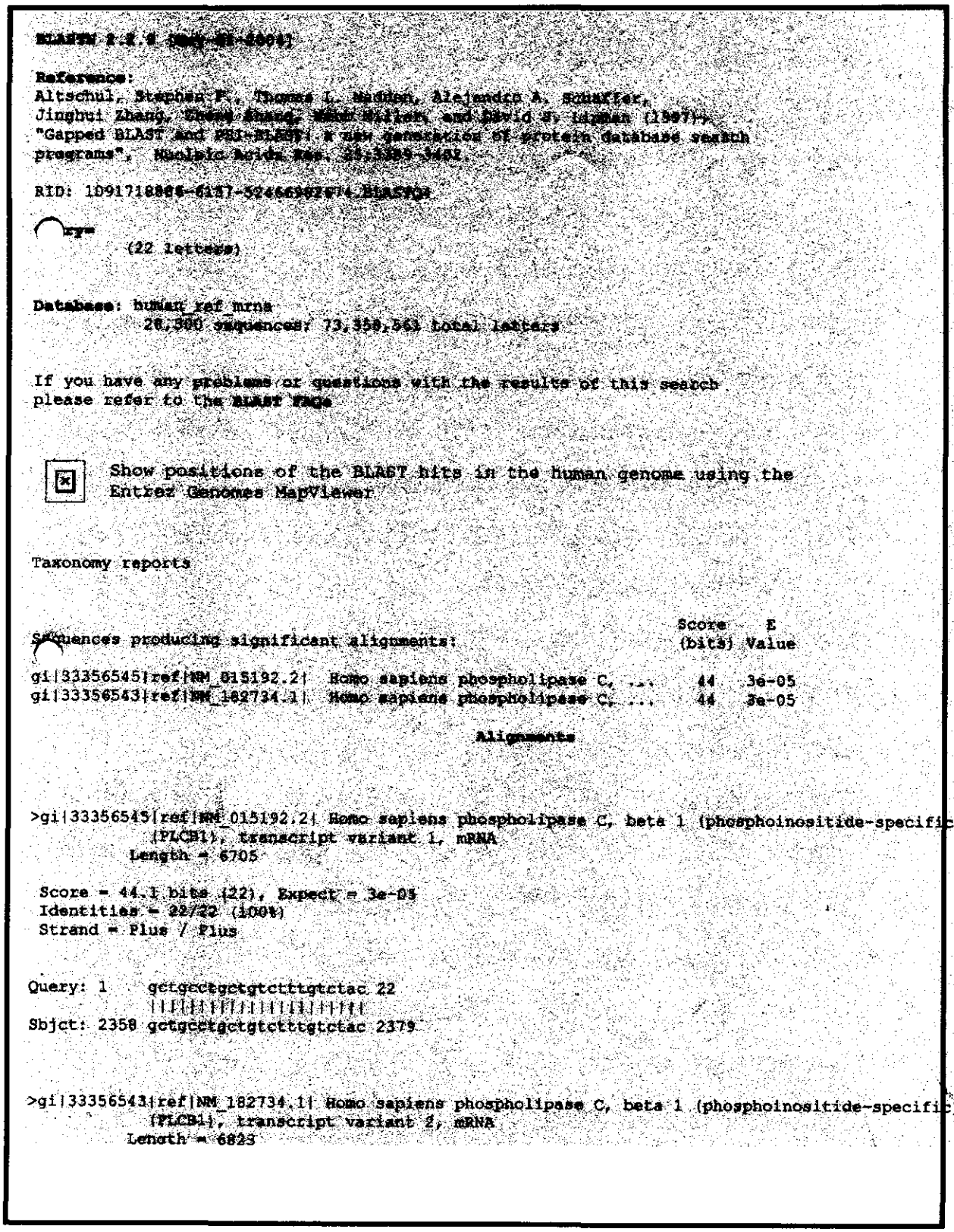
```
LOCUS       BC069420                3663 bp    mRNA    linear   PRI 19-AUG-
2004
DEFINITION  Homo sapiens phospholipase C, beta 1 (phosphoinositide-
specific),
            transcript variant 1, mRNA (cDNA clone MGC:97162
IMAGE:7262405),complete cds
ACCESSION   BC069420
VERSION     BC069420.1  GI:47480954
KEYWORDS    MGC.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3663)
  AUTHORS   Strausberg,R.L., Feingold,E.A., Grouse,L.H., Derge,J.G.,
            Klausner,R.D., Collins,F.S., Wagner,L., Shenmen,C.M.,
Schuler,G.D.,
            Altschul,S.F., Zeeberg,B., Buetow,K.H., Schaefer,C.F.,
Bhat,N.K.,
            Hopkins,R.F., Jordan,H., Moore,T., Max,S.I., Wang,J., Hsieh,F.,
            Diatchenko,L., Marusina,K., Farmer,A.A., Rubin,G.M., Hong,L.,
            Stapleton,M., Soares,M.B., Bonaldo,M.F., Casavant,T.L.,
            Scheetz,T.E., Brownstein,M.J., Usdin,T.B., Toshiyuki,S.,
            Carninci,P., Prange,C., Raha,S.S., Loquellano,N.A.,
Peters,G.J.,
            Abramson,R.D., Mullahy,S.J., Bosak,S.A., McEwan,P.J.,
            McKernan,K.J., Malek,J.A., Gunaratne,P.H., Richards,S.,
            Worley,K.C., Hale,S., Garcia,A.M., Gay,L.J., Hulyk,S.W.,
            Villalon,D.K., Muzny,D.M., Sodergren,E.J., Lu,X., Gibbs,R.A.,
            Fahey,J., Helton,E., Ketteman,M., Madan,A., Rodrigues,S.,
            Sanchez,A., Whiting,M., Madan,A., Young,A.C., Shevchenko,Y.,
            Bouffard,G.G., Blakesley,R.W., Touchman,J.W., Green,E.D.,
            Dickson,M.C., Rodriguez,A.C., Grimwood,J., Schmutz,J.,
Myers,R.M.,
            Butterfield,Y.S., Krzywinski,M.I., Skalska,U., Smailus,D.E.,
            Schnerch,A., Schein,J.E., Jones,S.J. and Marra,M.A.
  TITLE     Generation and initial analysis of more than 15,000 full-length
            human and mouse cDNA sequences
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)
   PUBMED   12477932
REFERENCE   2  (bases 1 to 3663)
  AUTHORS   Director MGC Project.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-APR-2004) National Institutes of Health,
Mammalian
```

```
            Gene Collection (MGC), Cancer Genomics Office, National Cancer
            Institute, 31 Center Drive, Room 11A03, Bethesda, MD 20892-
2590,
            USA
  REMARK    NIH-MGC Project URL: http://mgc.nci.nih.gov
COMMENT     Contact: MGC help desk
            Email: cgapbs-r@mail.nih.gov
            Tissue Procurement: Baylor Human Genome Sequencing Center
            cDNA Library Preparation: Baylor Human Genome Sequencing Center
            cDNA Library Arrayed by: The I.M.A.G.E. Consortium (LLNL)
            DNA Sequencing by: Baylor College of Medicine Human Genome
            Sequencing Center
            Center code: BCM-HGSC
            Web site: http://www.hgsc.bcm.tmc.edu/cdna/
            Contact: amg@bcm.tmc.edu
            Gunaratne, P.H., Garcia, A.M., Lu, X., Hulyk, S.W., Loulseged,
H.,
            Kowis, C.R., Sneed, A.J., Martin, R.G., Muzny, D.M., Nanavati,
            A.N., Gibbs, R.A.

            Clone distribution: MGC clone distribution information can be
found
            through the I.M.A.G.E. Consortium/LLNL at:
http://image.llnl.gov
            Series: IRBR Plate: 4 Row: c Column: 3.
FEATURES             Location/Qualifiers
     source          1..3663
                     /organism="Homo sapiens"
                     /mol_type="mRNA"
                     /db_xref="taxon:9606"
                     /clone="MGC:97162 IMAGE:7262405"
                     /tissue_type="PCR rescued clones"
                     /clone_lib="NIH_MGC_277"
                     /note="Vector: pPCR-Script Amp SK(+) with reversed
insert"
     gene            1..3663
                     /gene="PLCB1"
                     /note="synonyms: PI-PLC, PLC-154, PLC-I"
                     /db_xref="LocusID:23236"
                     /db_xref="MIM:607120"
     CDS             4..3654
                     /gene="PLCB1"
                     /codon_start=1
                     /product="phosphoinositide-specific phospholipase C
beta
                     1, isoform a"
                     /protein_id="AAH69420.1"
                     /db_xref="GI:47480955"
                     /db_xref="LocusID:23236"
                     /db_xref="MIM:607120"

/translation="MAGAQPGVHALQLKPVCVSDSLKKGTKFVKWDDDSTIVTPIILR

TDPQGFFFYWTDQNKETELLDLSLVKDARCGRHAKAPKDPKLRELLDVGNIGRLEQRM

ITVVYGPDLVNISHLNLVAFQEEVAKEWTNEVFSLATNLLAQNMSRDAFLEKAYTKLK

LQVTPEGRIPLKNIYRLFSADRKRVETALEACSLPSSRNDSIPQEDFTPEVYRVFLNN

LCPRPEIDNIFSEFGAKSKPYLTVDQMMDFINLKQRDPRLNEILYPPLKQEQVQVLIE
```

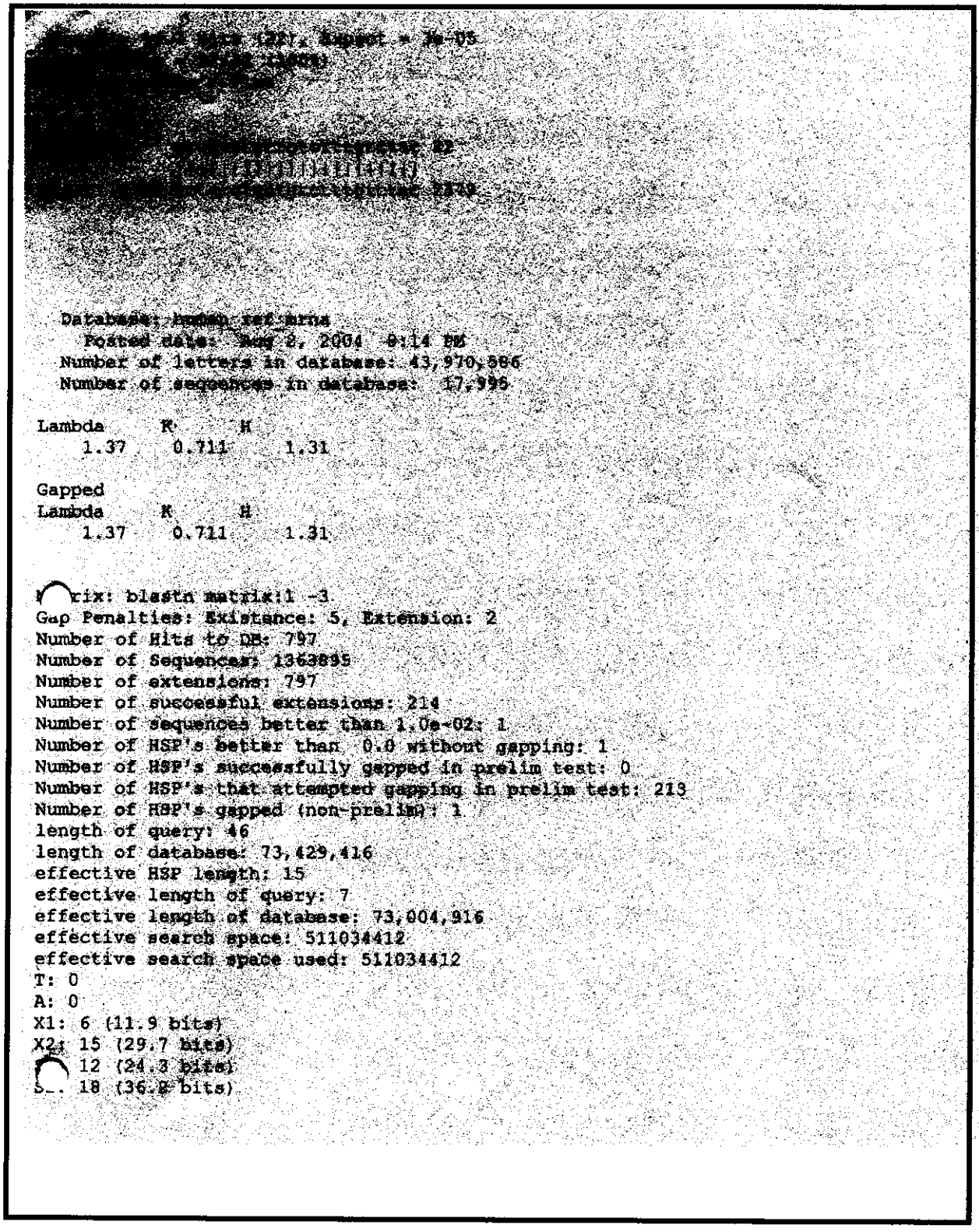
```
KYEPNNSLARKGQISVDGFMRYLSGEENGVVSPEKLDLNEDMSQPLSHYFINSSHNTY

LTAGQLAGNSSVEMYRQVLLSGCRCVELDCWKGRTAEEEPVITHGFTMTTEISFKEVI

EAIAECAFKTSPFPILLSFENHVDSPKQQAKMAEYCRLIFGDALLMEPLEKYPLESGV

PLPSPMDLMYKILVKNKKKSHKSSEGSGKKKLSEQASNTYSDSSSMFEPSSPGAGEAD

TESDDDDDDDDCKKSSMDEGTAGSEAMATEEMSNLVNYIQPVKFESFEISKKRNKSFE

MSSFVETKGLEQLTKSPVEFVEYNKMQLSRIYPKGTRVDSSNYMPQLFWNAGCQMVAL

NFQTMDLAMQINMGMYEYNGKSGYRLKPEFMRRPDKHFDPFTEGIVDGIVANTLSVKI

ISGQFLSDKKVGTYVEVDMFGLPVDTRRKAFKTKTSQGNAVNPVWEEEPIVFKKVVLP

TLACLRIAVYEEGGKFIGHRILPVQAIRPGYHYICLRNERNQPLTLPAVFVYIEVKDY

VPDTYADVIEALSNPIRYVNLMEQRAKQLAALTLEDEEEVKKEADPGETPSEAPSEAR

TTPAENGVNHTTTLTPKPPSQALHSQPAPGSVKAPAKTEDLIQSVLTEVEAQTIEELK

QQKSFVKLQKKHYKEMKDLVKRHHKKTTDLIKEHTTKYNEIQNDYLRRRAALEKSAKK

DSKKKSEPSSPDHGSSTIEQDLAALDAEMTQKLIDLKDKQQQQLLNLRQEQYYSEKYQ

KREHIKLLIQKLTDVAEECQNNQLKKLKEICEKEKKELKKKMDKKRQEKITEAKSKDK

SQMEEEKTEMIRSYIQEVVQYIKRLEEAQSKRQEKLVEKHKEIRQQILDEKPKLQVEL

EQEYQDKFKRLPLEILEFVQEAMKGKISEDSNHGSAPLSLSSDPGKVNHKTPSSEELG
                    GDIPGKEFDTPL"
     misc difference 2991
                    /gene="PLCB1"
                    /note="'C' in cDNA is 'T' in the human genome; no
amino
                    acid change."
ORIGIN
        1 cagatggccg gggctcaacc cggagtgcac gccttgcaac tcaagcccgt gtgcgtgtcc
       61 gacagcctca agaagggcac caaattcgtc aagtgggatg atgactcaac tattgttact
      121 ccaattattt tgaggactga ccctcaggga tttttctttt actggacaga tcaaaacaag
      181 gagacagagc tactggatct cagccttgtc aaagatgcca gatgtgggag acacgccaaa
      241 gctcccaagg accccaaatt acgtgaactt ttggatgtgg ggaacatcgg gcgcctggag
      301 cagcgcatga tcacagtggt gtatgggcct gacctcgtga acatctccca tttgaatctc
      361 gtggctttcc aagaagaagt ggccaaggaa tggacaaatg aggttttcag tttggcaaca
      421 aacctgctgg cccaaaacat gtccagggat gcatttctgg aaaaagccta tactaaactt
      481 aagctgcaag tcactccaga agggcgtatt cctctcaaaa acatatatcg cttgttttca
      541 gcagatcgga agcgagttga aactgcttta gaggcttgta gtcttccatc ttcaaggaat
      601 gattcaatac ctcaagaaga tttcactcca gaagtgtaca gagttttcct caacaacctt
      661 tgccctcgac ctgaaattga taacatcttt tcagaatttg gtgcaaaaag caaaccatat
      721 cttaccgttg atcagatgat ggattttatc aaccttaagc agcgagatcc tcggcttaat
      781 gaaatacttt atccacctct aaaacaagag caagtccaag tattgattga gaagtatgaa
      841 cccaacaaca gcctcgccag aaaaggacaa atatcagtgg atgggttcat gcgctatctg
      901 agtggagaag aaaacggagt cgtttcacct gagaaactgg atttgaatga agacatgtct
      961 cagccccttt ctcactattt cattaattcc tcgcacaaca cctacctcac agctggccaa
     1021 ctggctggaa actcctctgt tgagatgtat cgccaagtgc tcctgtctgg ttgtcgctgt
     1081 gtggagctgg actgctggaa gggacggact gcagaagagg aacctgtcat cacccatggc
     1141 ttcaccatga caactgaaat atctttcaag gaagtgatag aagcaattgc ggagtgtgca
     1201 tttaagactt caccttttcc aattctcctt tcgtttgaga accatgtgga ttccccaaag
```

```
1261 cagcaagcca agatggcgga gtactgccga ctgatctttg gggatgccct tctcatggag
1321 cccctggaaa aatatccact ggaatctgga gttcctcttc caagccctat ggatttaatg
1381 tataaaattt tggtgaaaaa taagaagaaa tcacacaagt catcagaagg aagcggcaaa
1441 aagaagctct cagaacaagc ctccaacacc tacagtgact cctccagcat gttcgagccc
1501 tcatccccag gagccggaga agctgatacg gaaagtgacg acgacgatga tgatgatgac
1561 tgtaaaaaat cttcaatgga tgaggggact gctggaagtg aggctatggc cacagaagaa
1621 atgtctaatc tggtgaacta tattcagcca gtcaagtttg agtcatttga aatttcaaaa
1681 aaaagaaata aaagttttga aatgtcttcc ttcgtggaaa ccaaaggact tgaacaactc
1741 accaagtctc cagtggaatt tgtagaatat aacaaaatgc agcttagcag gatatatcca
1801 aaaggaacac gtgtggattc atccaactat atgcctcagc tcttctggaa tgcaggttgt
1861 cagatggtgg cacttaattt ccagacaatg gacctggcta tgcaaataaa tatggggatg
1921 tatgaataca acgggaagag tggctacaga ttgaagccag agttcatgag gaggcctgac
1981 aagcattttg atccatttac tgaaggcatc gtagatggga tagtggcaaa cactttgtct
2041 gttaagatta tttcaggtca gtttctttct gataagaaag ttgggactta cgtggaagta
2101 gatatgtttg gtttgcctgt ggatacaagg aggaaggcat ttaagaccaa aacatcccaa
2161 ggaaatgctg tgaatcctgt ctgggaagaa gaacctattg tgttcaaaaa ggtggttctt
2221 cctactctgg cctgtttgag aatagcagtt tatgaagaag gaggtaaatt cattggccac
2281 cgtatcttgc cagtgcaagc cattcggcca ggctatcact atatctgtct aaggaatgaa
2341 aggaaccagc ctctgacgct gcctgctgtc tttgtctaca tagaagtgaa agactatgtg
2401 ccagacacat atgcagatgt catcgaagct ttatcaaacc caatccgata tgtgaacctg
2461 atggaacaga gagctaagca attggctgct ttgacactgg aagatgaaga agaagtaaag
2521 aaagaggctg atcctggaga aacaccatca gaggctccaa gtgaagcgag aacgactcca
2581 gcagaaaatg gggtgaatca cactacaacc ctgacaccca agccaccctc ccaggctctc
2641 cacagccagc cagctccagg ttctgtaaag gcacctgcca aaacagaaga tcttattcag
2701 agtgtcttaa cagaagtgga agcacagacc atcgaagaac taaagcaaca gaaatcgttt
2761 gtgaaacttc aaaagaaaca ctacaaagaa atgaaagacc tggttaagag acaccacaag
2821 aaaaccactg accttatcaa agaacacact accaagtata atgaaattca gaatgactac
2881 ttgagaagga gagccgcttt ggaaaagtcc gccaaaaagg acagtaagaa aaaatcggaa
2941 cccagcagcc ctgatcatgg ttcatcaacg attgagcaag acctcgctgc cctggatgct
3001 gaaatgaccc aaaagttaat agacttgaag gacaaacaac agcagcagct gcttaatctt
3061 cggcaagaac agtattatag tgaaaaatac cagaagcgag aacatattaa actgcttatt
3121 caaaagttga cggatgtcgc agaagagtgt cagaacaatc agttaaagaa gctcaaagaa
3181 atctgtgaga aagaaaagaa agaattaaag aagaaaatgg ataaaaagag gcaggagaag
3241 ataacagaag ctaaatccaa agacaaaagt cagatggaag aggagaagac agagatgatc
3301 cggtcatata tccaggaagt ggtgcagtat atcaagaggc tagaagaagc gcaaagtaaa
3361 cggcaagaaa aactcgtaga gaaacacaag gaaatacgtc agcagatcct ggatgaaaag
3421 cccaagctgc aggtggagct ggagcaagaa taccaagaca aattcaaaag actgcccctc
3481 gagattttgg aattcgtgca ggaagccatg aaaggaaaga tcagtgaaga cagcaatcac
3541 ggttctgccc ctctctccct gtcctcagac cctggaaaag tgaaccacaa gactccctcc
3601 agtgaggagc tgggaggaga catcccagga aaagaatttg atactcctct gtgaatgctc
3661 ctg
```

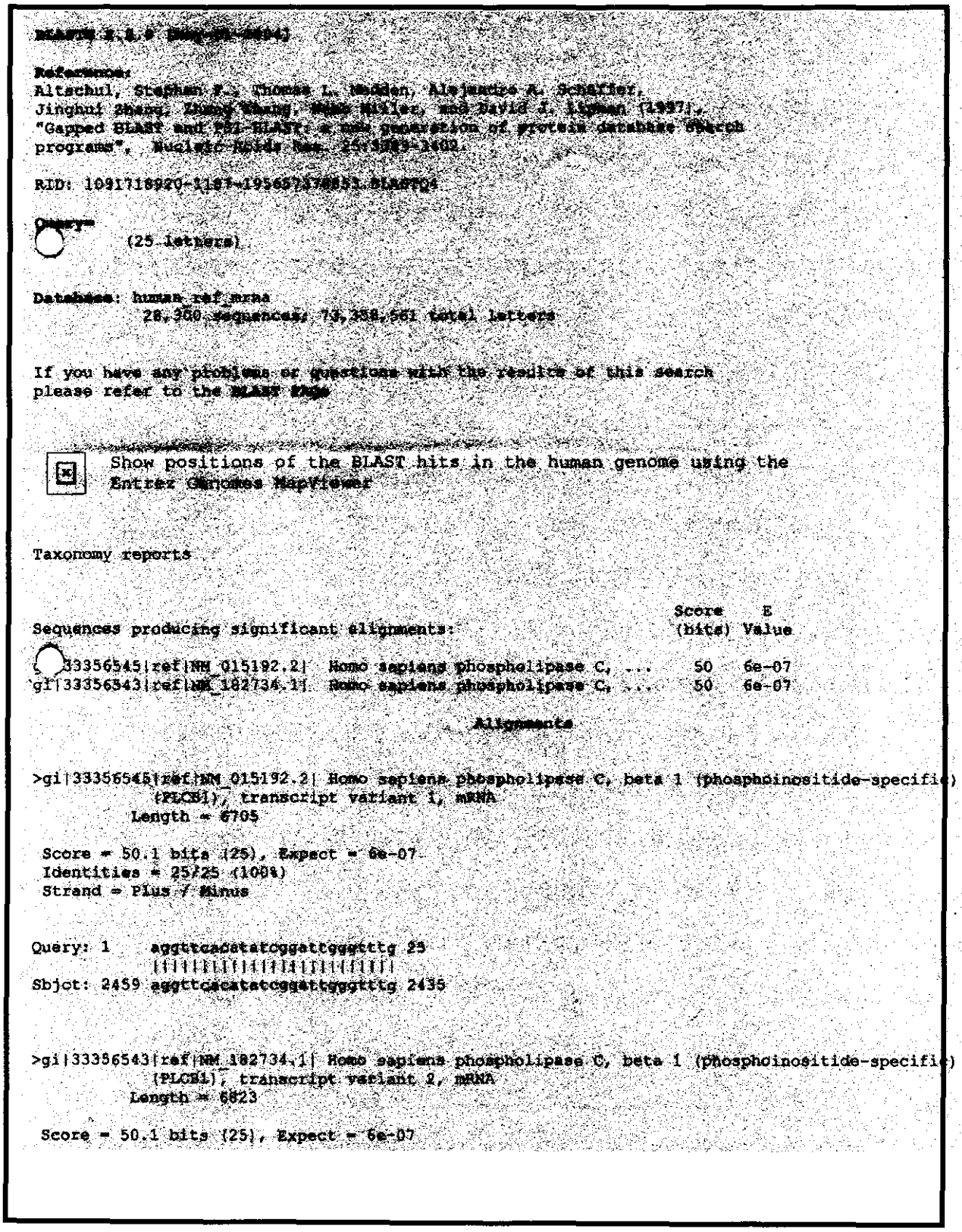
B.2 Blast results



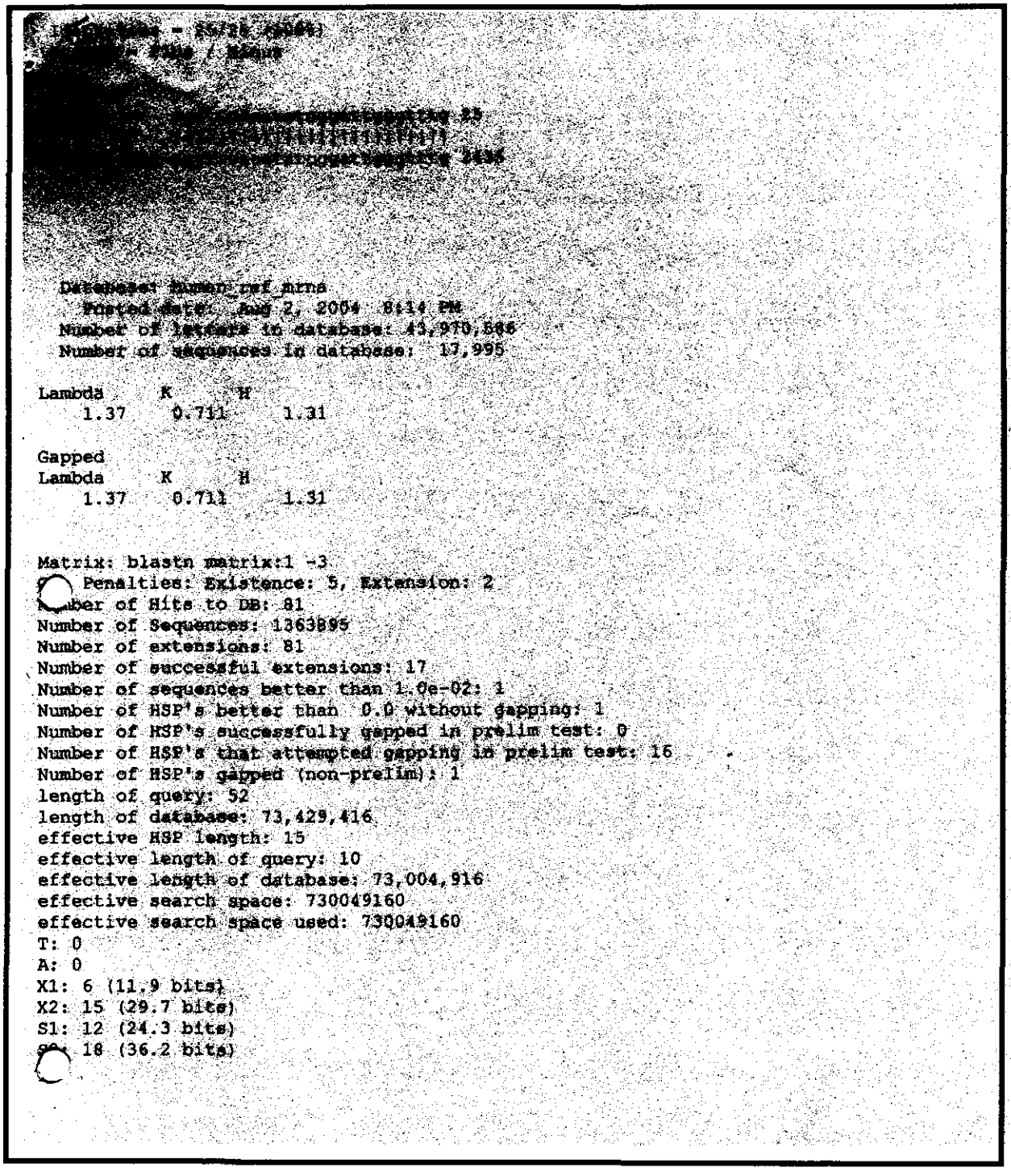
```
BLASTN 2.2.6 [Sep-21-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

RID: 1091718866-6187-52466902674.BLASTQ1

Query=
      (22 letters)

Database: human_ref_mrna
           20,300 sequences; 73,358,563 total letters

If you have any problems or questions with the results of this search
please refer to the BLAST FAQs

  Show positions of the BLAST hits in the human genome using the
  Entrez Genomes MapViewer

Taxonomy reports

                                                                  Score     E
Sequences producing significant alignments:                       (bits) Value

gi|33356545|ref|NM_015192.2|  Homo sapiens phospholipase C, ...    44   3e-05
gi|33356543|ref|NM_182734.1|  Homo sapiens phospholipase C, ...    44   3e-05

                                Alignments


>gi|33356545|ref|NM_015192.2| Homo sapiens phospholipase C, beta 1 (phosphoinositide-specific)
           (PLCB1), transcript variant 1, mRNA
          Length = 6705

 Score = 44.1 bits (22), Expect = 3e-05
 Identities = 22/22 (100%)
 Strand = Plus / Plus


Query: 1    gctgcctgctgtctttgtctac 22
             ||||||||||||||||||||||
Sbjct: 2358 gctgcctgctgtctttgtctac 2379


>gi|33356543|ref|NM_182734.1| Homo sapiens phospholipase C, beta 1 (phosphoinositide-specific)
           (PLCB1), transcript variant 2, mRNA
          Length = 6823
```



```
  Database: human_ref_mrna
    Posted date:  Aug 2, 2004  8:14 PM
  Number of letters in database: 43,970,586
  Number of sequences in database:  17,995

Lambda     K      H
    1.37   0.711     1.31

Gapped
Lambda     K      H
    1.37   0.711     1.31

Matrix: blastn matrix:1 -3
Gap Penalties: Existance: 5, Extension: 2
Number of Hits to DB: 797
Number of Sequences: 1363895
Number of extensions: 797
Number of successful extensions: 214
Number of sequences better than 1.0e-02: 1
Number of HSP's better than  0.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 213
Number of HSP's gapped (non-prelim): 1
length of query: 46
length of database: 73,429,416
effective HSP length: 15
effective length of query: 7
effective length of database: 73,004,916
effective search space: 511034412
effective search space used: 511034412
T: 0
A: 0
X1: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: 12 (24.3 bits)
S2: 18 (36.0 bits)
```



```
BLASTN 2.2.9 [Aug-26-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

RID: 1091718929-1187-195657178851.BLASTQ4

Query=
      (25 letters)

Database: human ref_mrna
           28,360 sequences; 73,358,561 total letters


If you have any problems or questions with the results of this search
please refer to the BLAST FAQs


  Show positions of the BLAST hits in the human genome using the
  Entrez Genomes MapViewer


Taxonomy reports


                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

 gi|33356545|ref|NM_015192.2|  Homo sapiens phospholipase C, ...    50   6e-07
gi|33356543|ref|NM_182734.1|  Homo sapiens phospholipase C, ...    50   6e-07

                                Alignments


>gi|33356545|ref|NM_015192.2| Homo sapiens phospholipase C, beta 1 (phosphoinositide-specific)
           (PLCB1), transcript variant 1, mRNA
          Length = 6705

 Score = 50.1 bits (25), Expect = 6e-07
 Identities = 25/25 (100%)
 Strand = Plus / Minus


Query: 1    aggttcacatatcggattgggtttg 25
             |||||||||||||||||||||||||
Sbjct: 2459 aggttcacatatcggattgggtttg 2435


>gi|33356543|ref|NM_182734.1| Homo sapiens phospholipase C, beta 1 (phosphoinositide-specific)
           (PLCB1), transcript variant 2, mRNA
          Length = 6823

 Score = 50.1 bits (25), Expect = 6e-07
```



```
Database: Human_ref_mrna
  Posted date:  Aug 2, 2004  8:14 PM
Number of letters in database: 43,970,566
Number of sequences in database:  17,995

Lambda     K      H
    1.37    0.711     1.31

Gapped
Lambda     K      H
    1.37    0.711     1.31


Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 81
Number of Sequences: 1363895
Number of extensions: 81
Number of successful extensions: 17
Number of sequences better than 1.0e-02: 1
Number of HSP's better than  0.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 16
Number of HSP's gapped (non-prelim): 1
length of query: 52
length of database: 73,429,416
effective HSP length: 15
effective length of query: 10
effective length of database: 73,004,916
effective search space: 730049160
effective search space used: 730049160
T: 0
A: 0
X1: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: 12 (24.3 bits)
S2: 18 (36.2 bits)
```

B.2.1 Temperature gradient for the designed primer oligos of the target genes

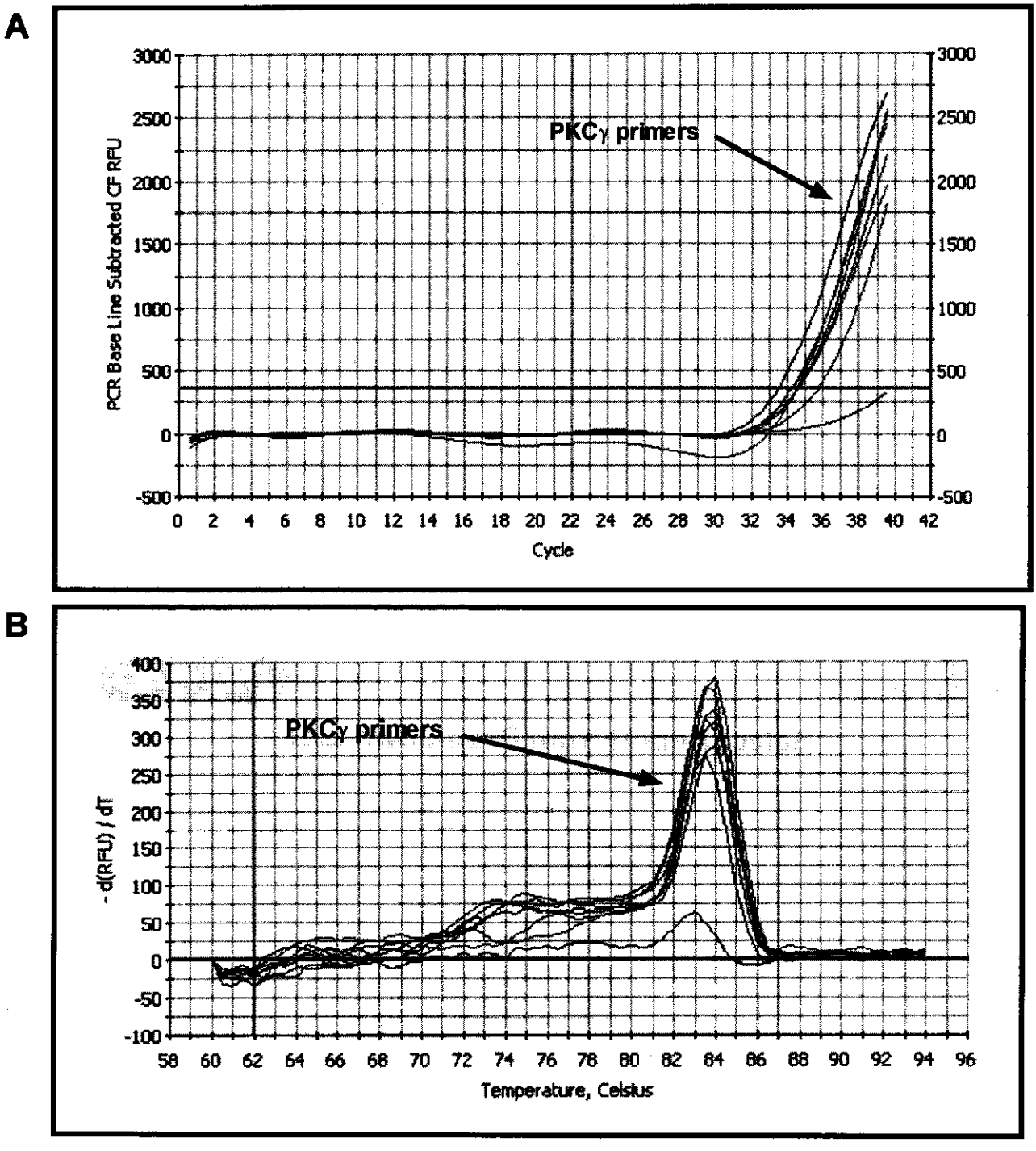


Figure B-1: *(A) Amplification curve of the cDNA for Protein Kinase Cγ after a temperature gradient was performed. (B) Melting curve of amplification product(s) with Protein Kinase Cγ primers after a temperature gradient was performed.*

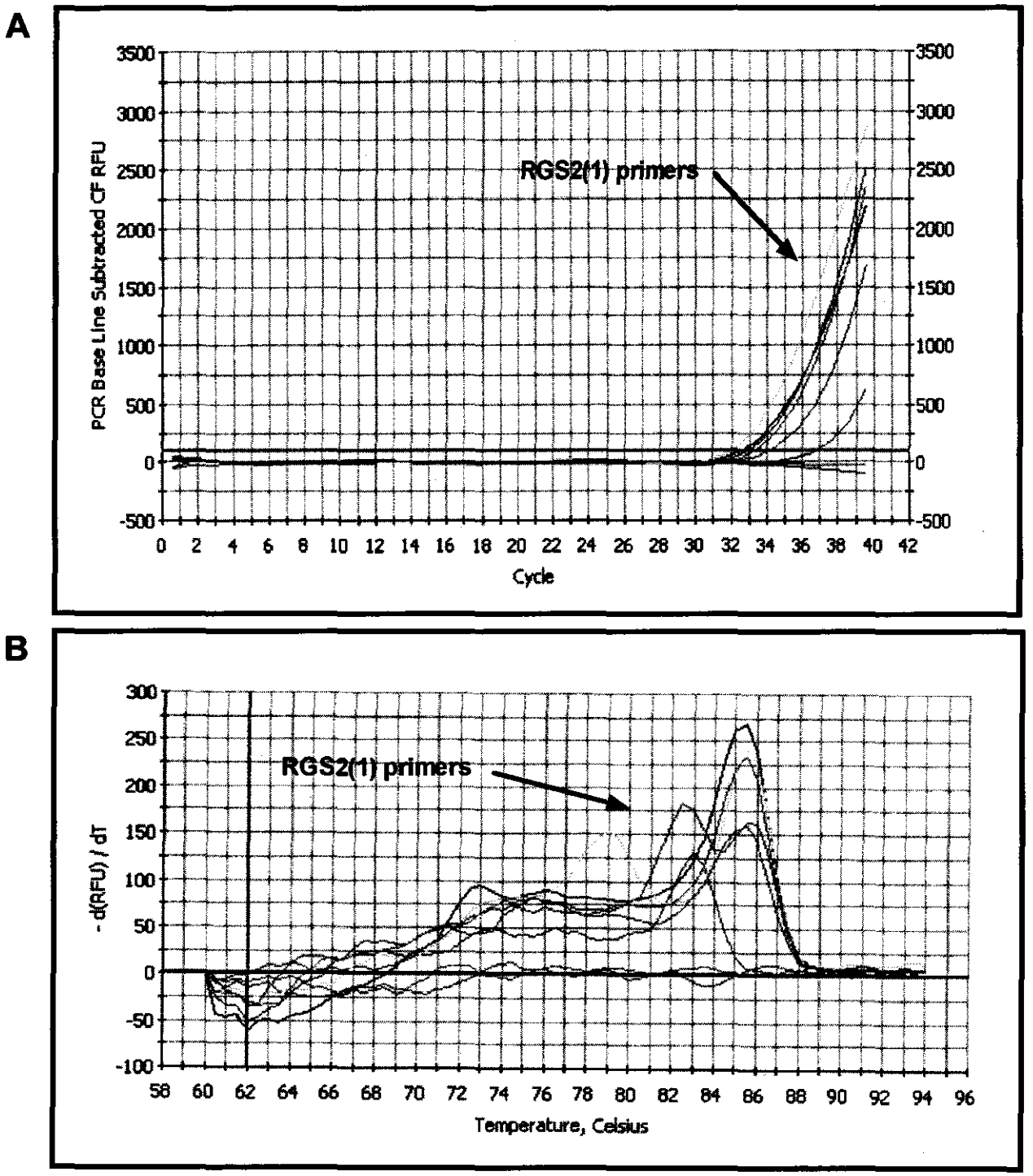


Figure B-2: *(A) Amplification curve of the cDNA for Regulators of G protein-coupled receptor signalling 2 (RGS2) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with RGS2 primers after a temperature gradient was performed.*

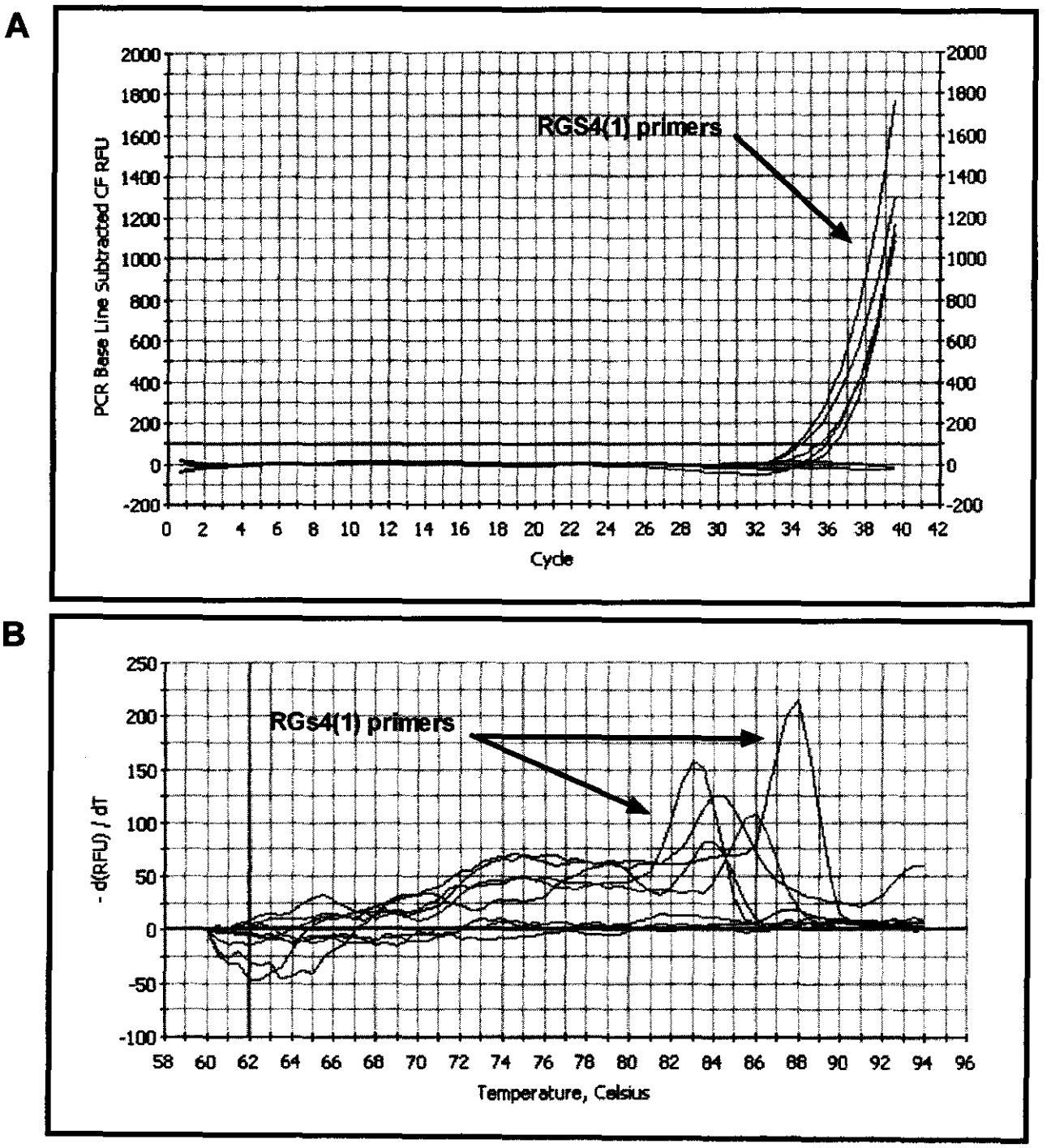


Figure B-3: *(A) Amplification curve of the cDNA for Regulators of G protein-coupled receptor signalling 4 (RGS4) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with RGS4 primers after a temperature gradient was performed.*

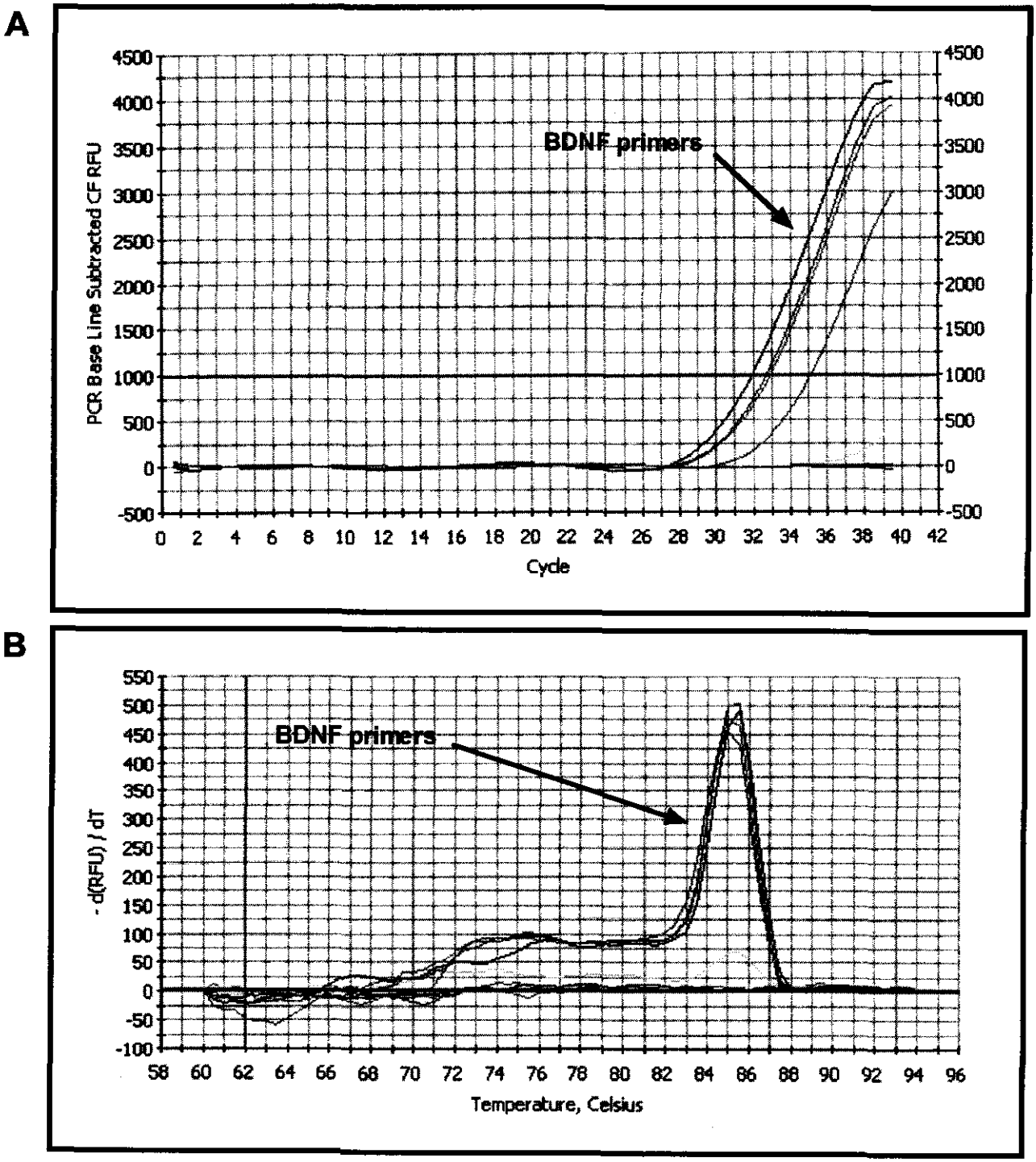


Figure B-4: *(A) Amplification curve of the cDNA for brain derived neurotrophic factor (BDNF) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with BDNF primers after a temperature gradient was performed.*

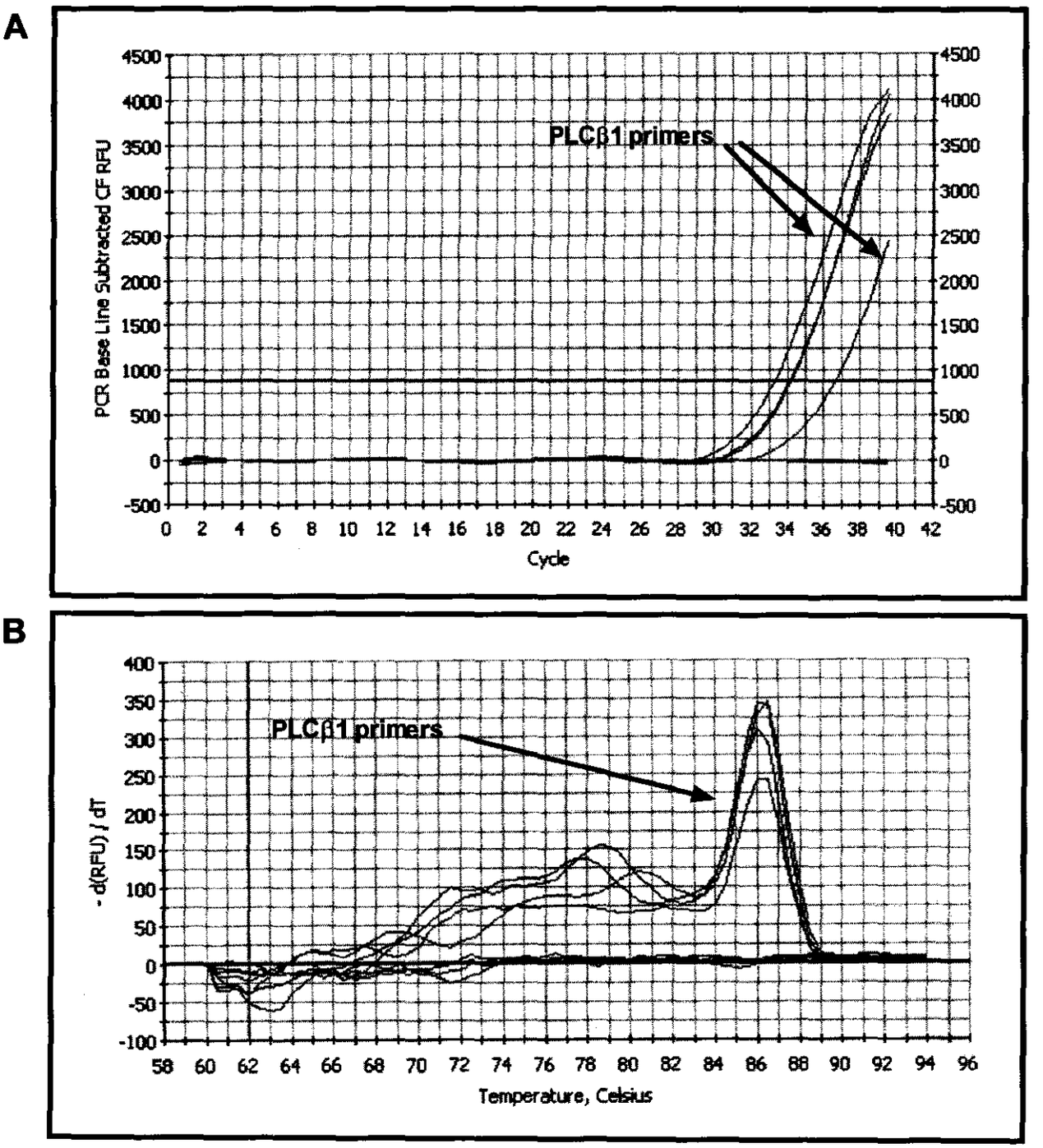


Figure B-5: *(A) Amplification curve of the cDNA for Phospholipase C β1 (PLCβ1) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with PLCβ1 primers after a temperature gradient was performed.*

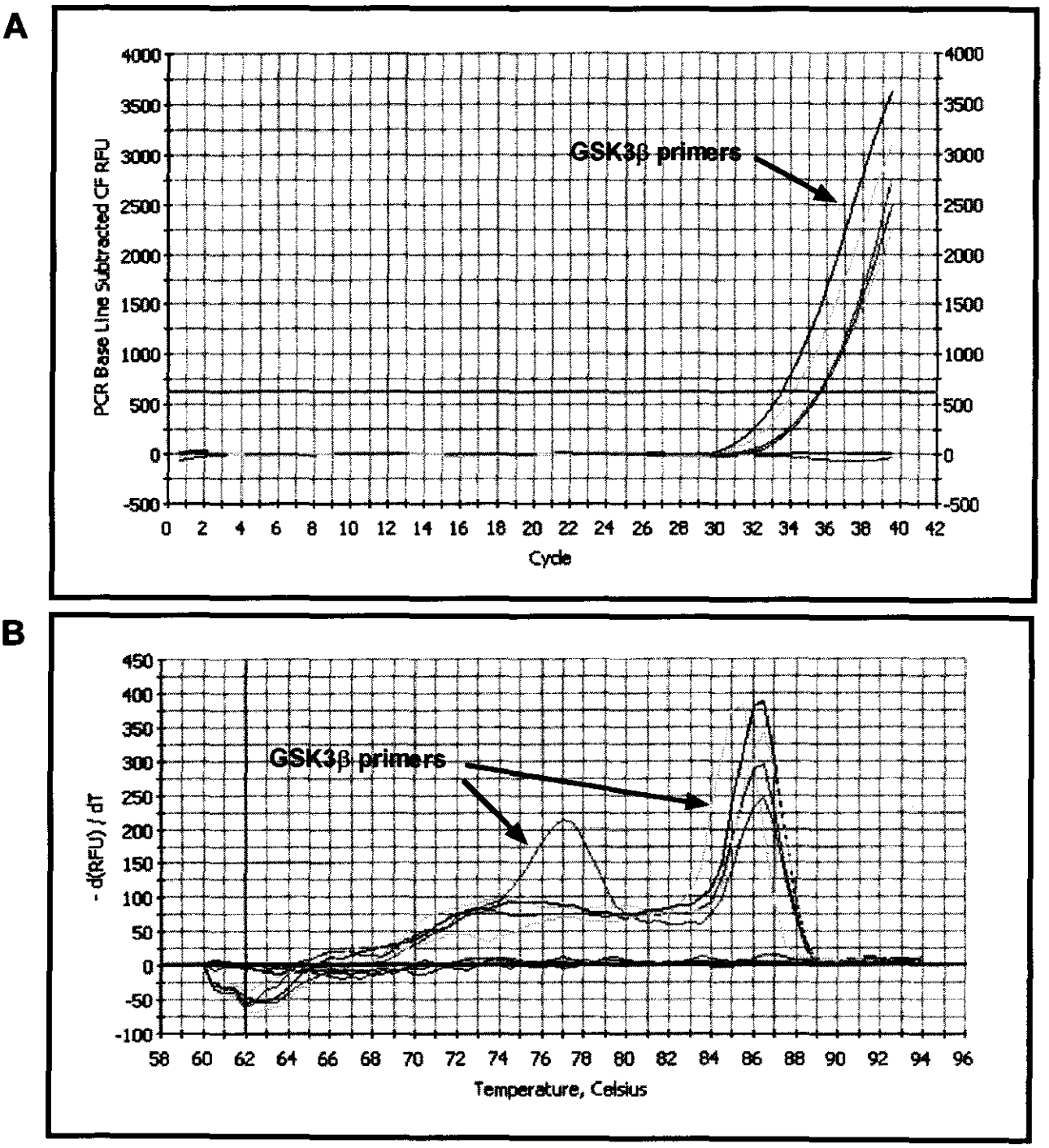


Figure B-6: *(A) Amplification curve of the cDNA for Glycogen synthase kinase 3β (GSK3β) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with GSK3β primers after a temperature gradient was performed.*

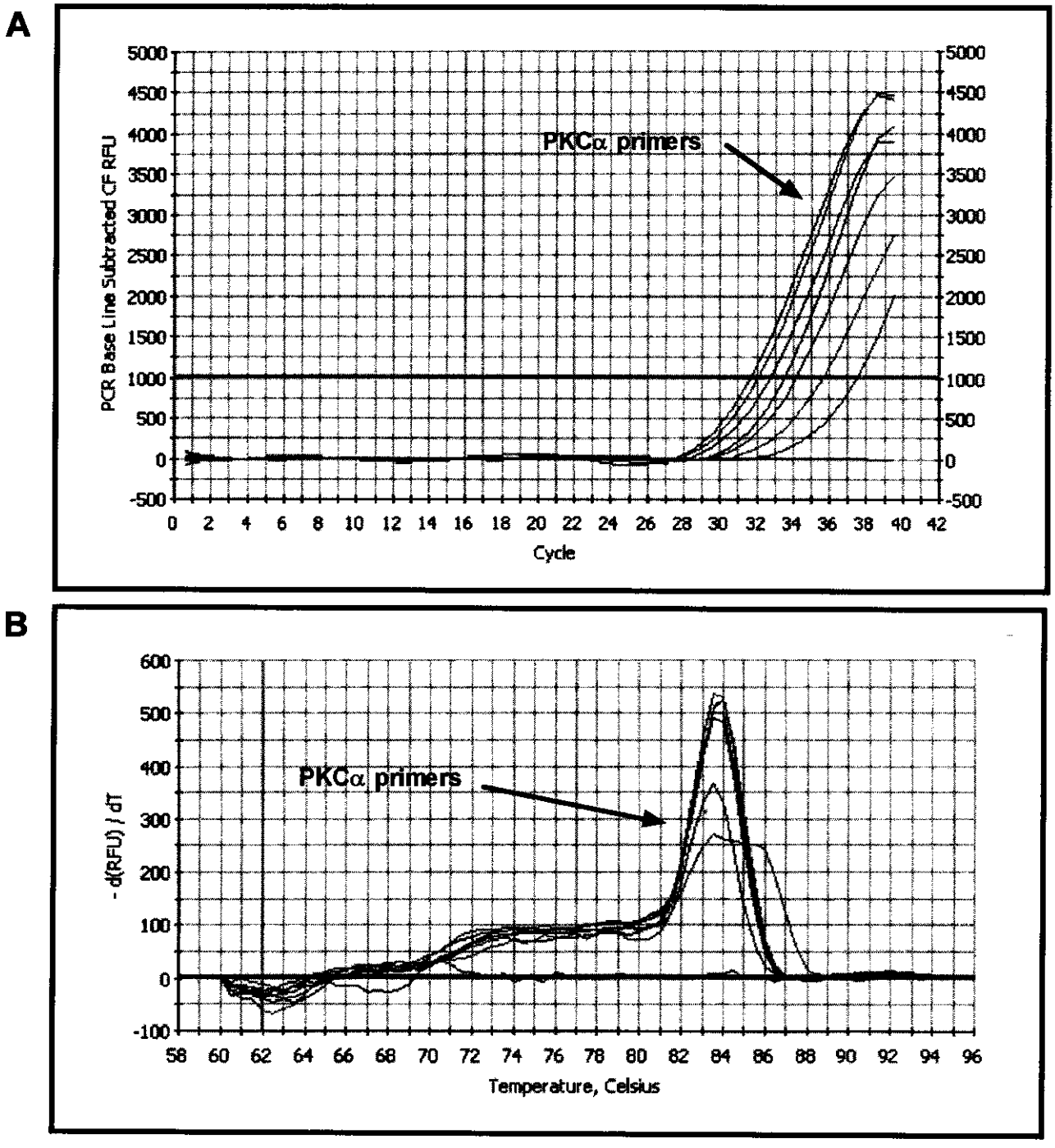


Figure B-7: *(A) Amplification curve of the cDNA for Protein Kinase Cα (PKCα) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with PKCα primers after a temperature gradient was performed.*

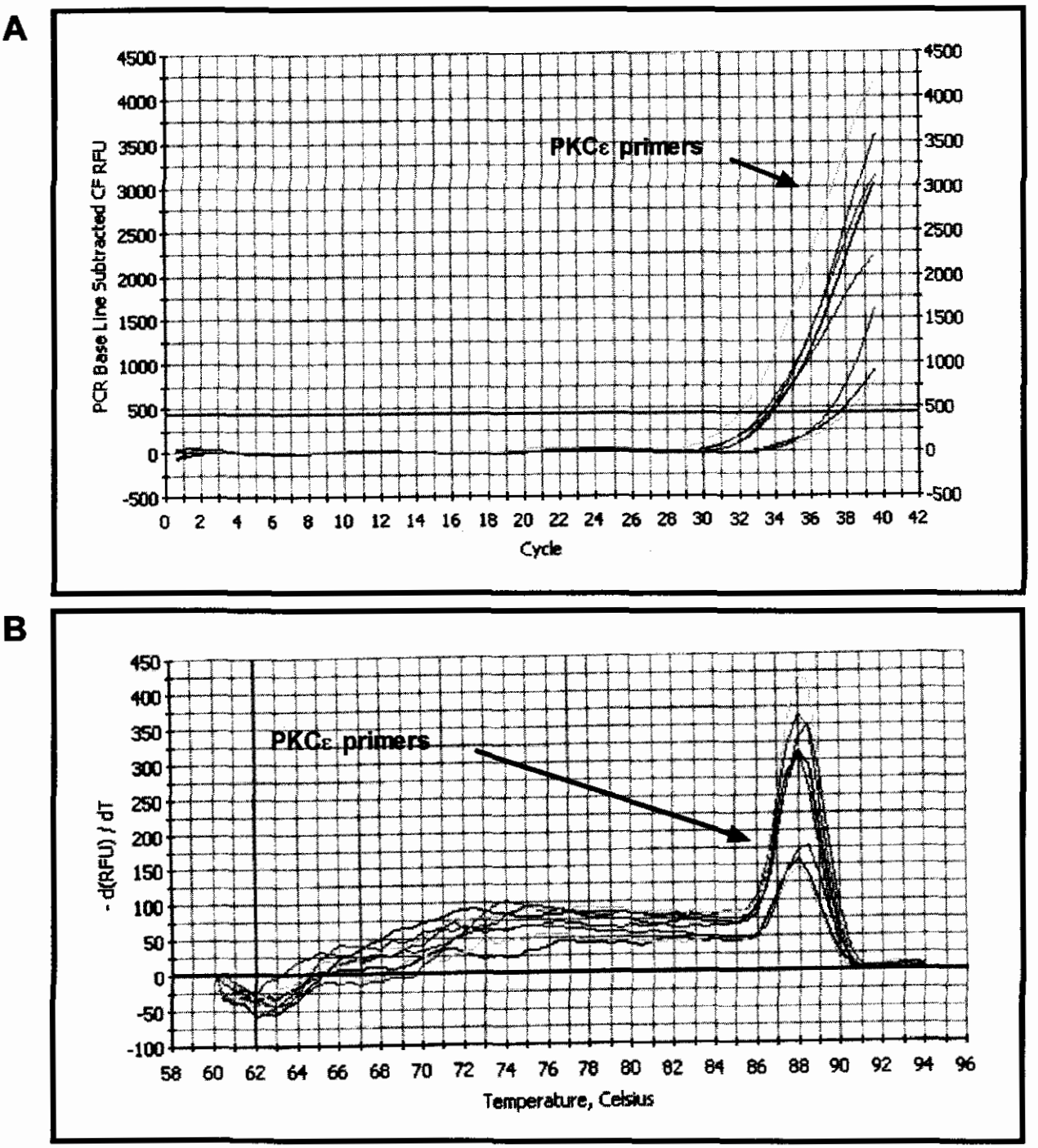


Figure B-8: *(A) Amplification curve of the cDNA for Protein Kinase Cε (PKCε) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with PKCε primers after a temperature gradient was performed.*

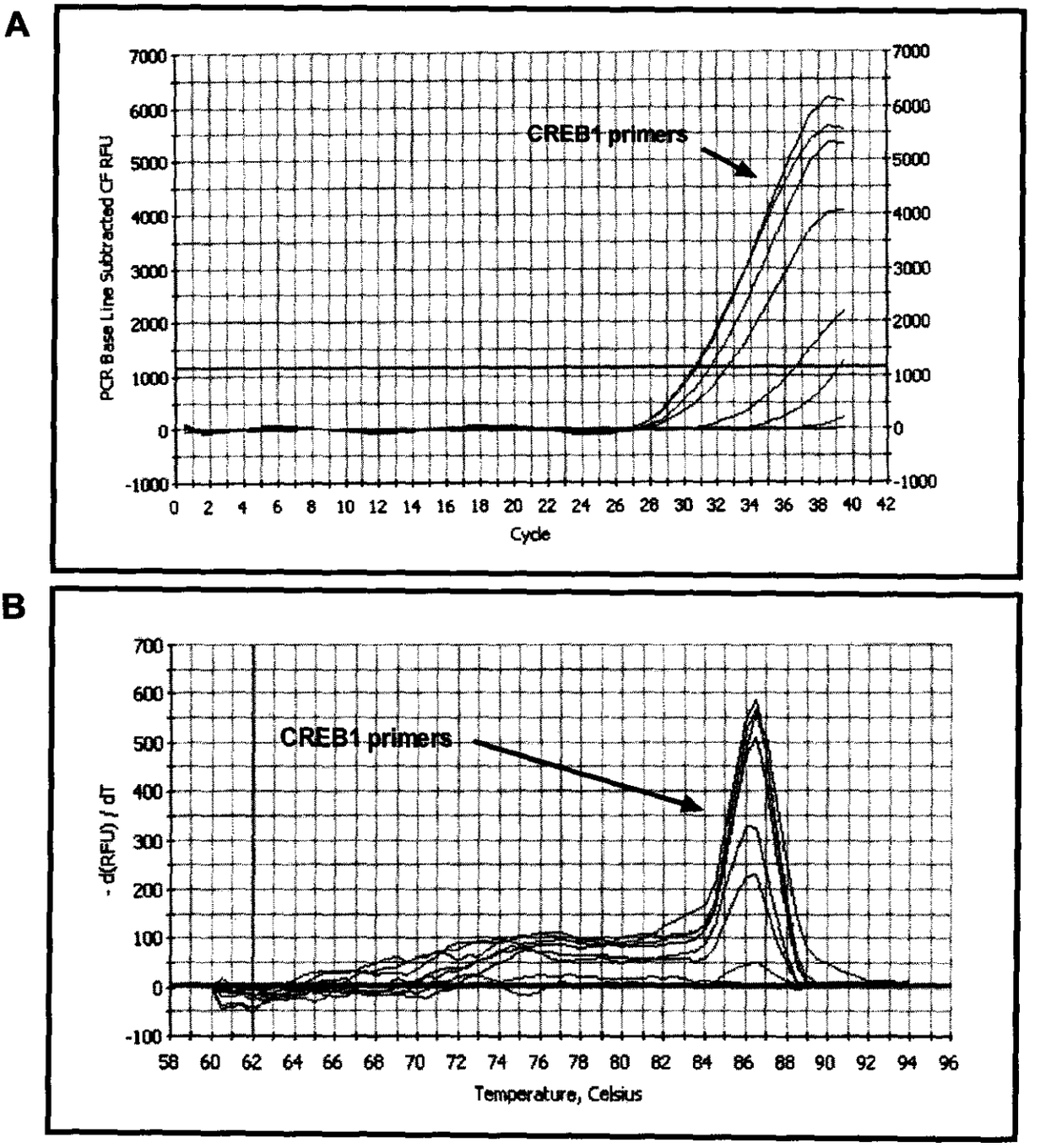


Figure B-9: *(A) Amplification curve of the cDNA for cAMP response element binding protein (CREB1) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with CREB1 primers after a temperature gradient was performed.*

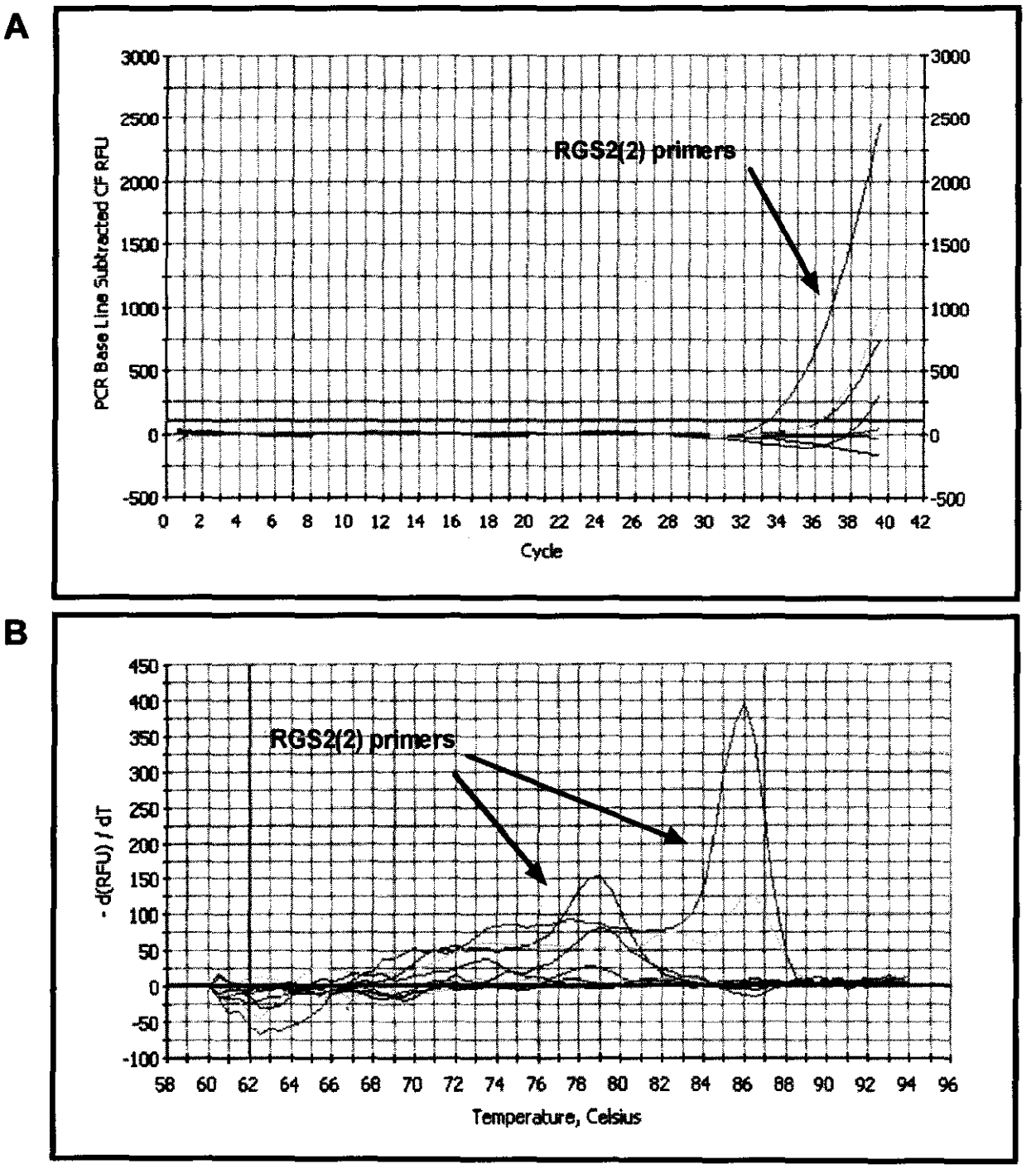


Figure B-10: *(A) Amplification curve of the cDNA for Regulators of G protein-coupled receptor signalling 2 (RGS2) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with RGS2 primers after a temperature gradient was performed.*

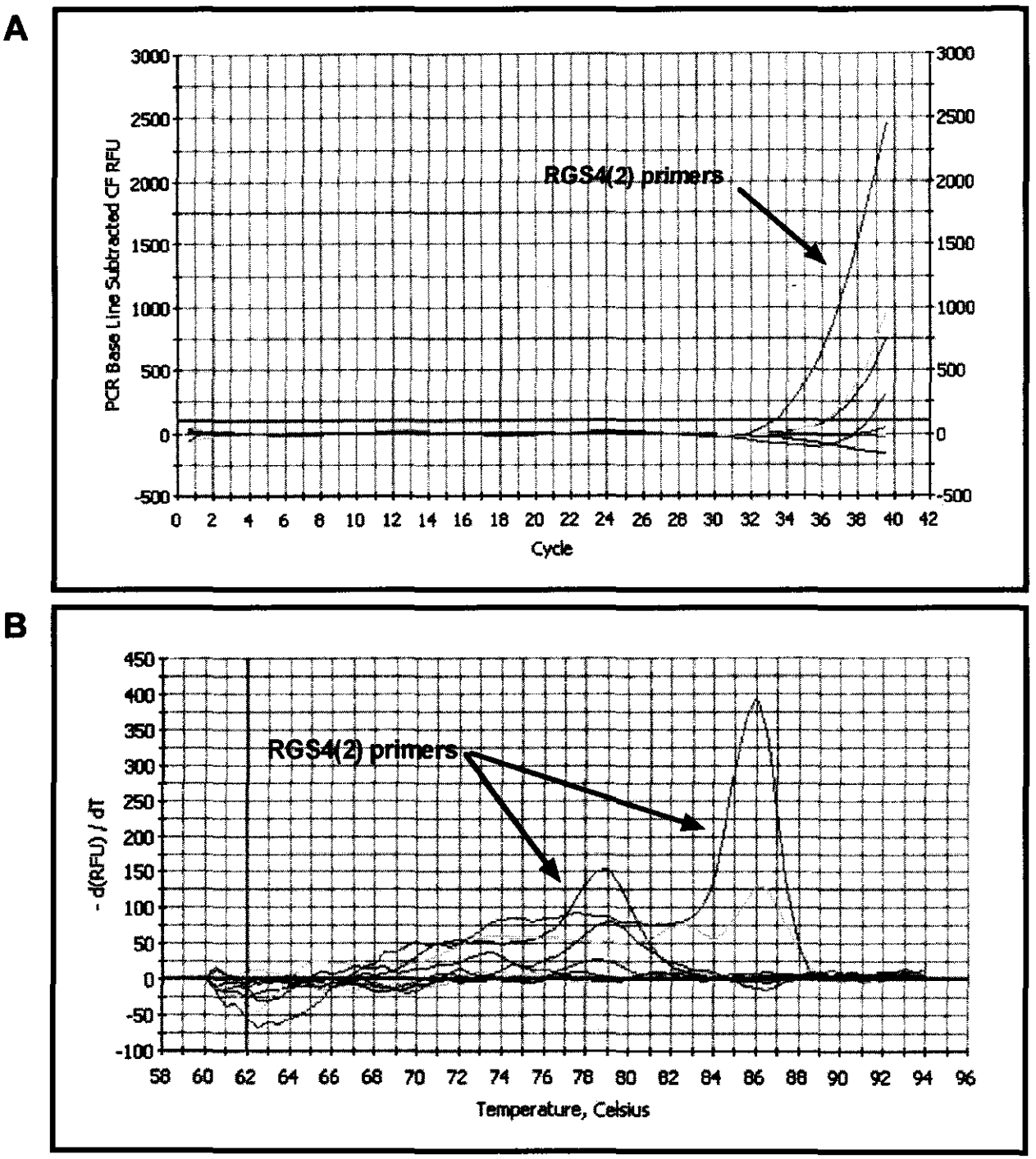


Figure B-11: *(A) Amplification curve of the cDNA for Regulators of G protein-coupled receptor signalling 4 (RGS4) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with RGS4 primers after a temperature gradient was performed.*

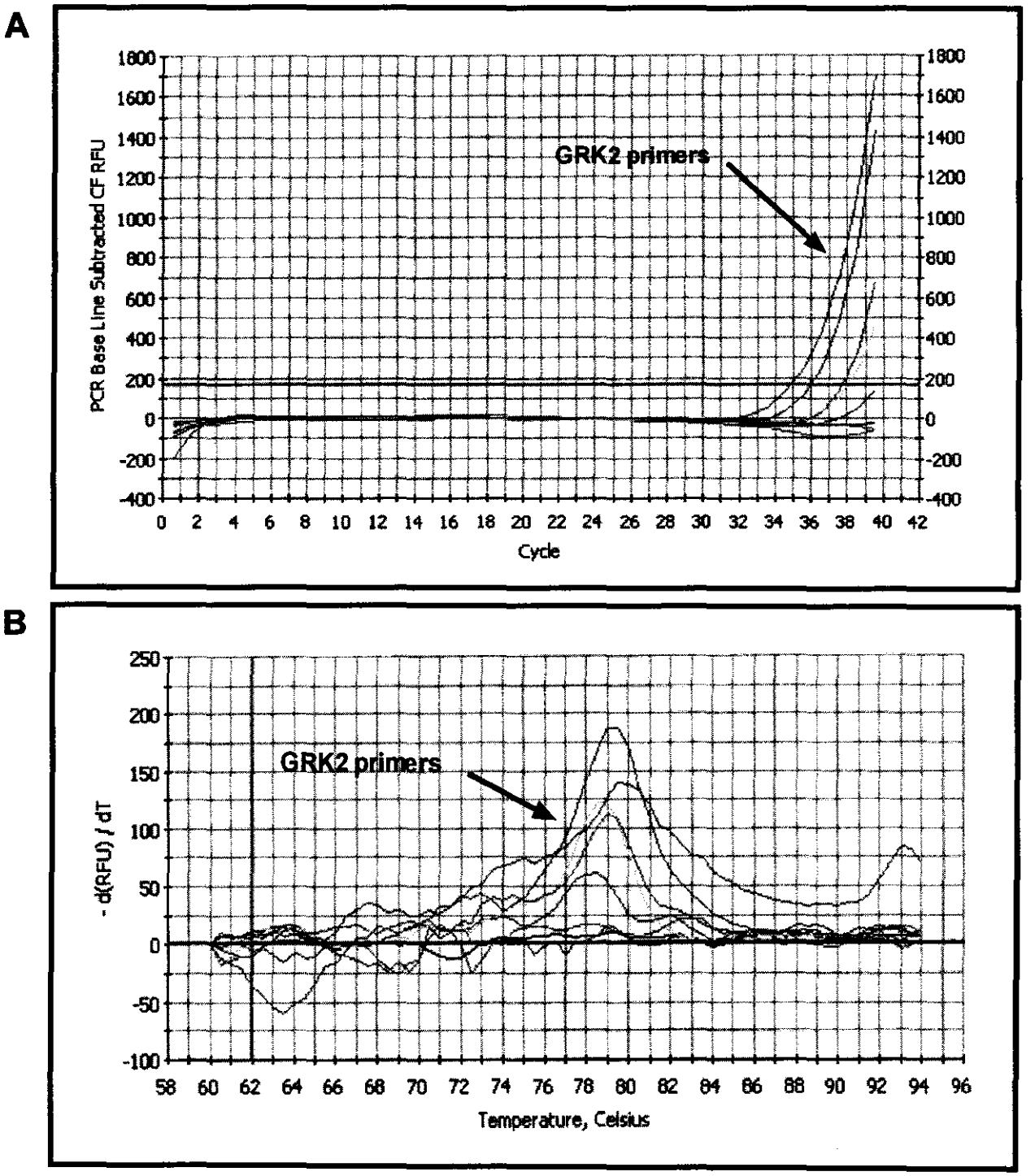


Figure B-12: *(A) Amplification curve of the cDNA for G-protein receptor kinase 2 (GRK2) after a temperature gradient was performed. (B) Melting curve of amplification product(s) with GRK2 primers after a temperature gradient was performed.*

B.2.2 Real-time RT-PCR results

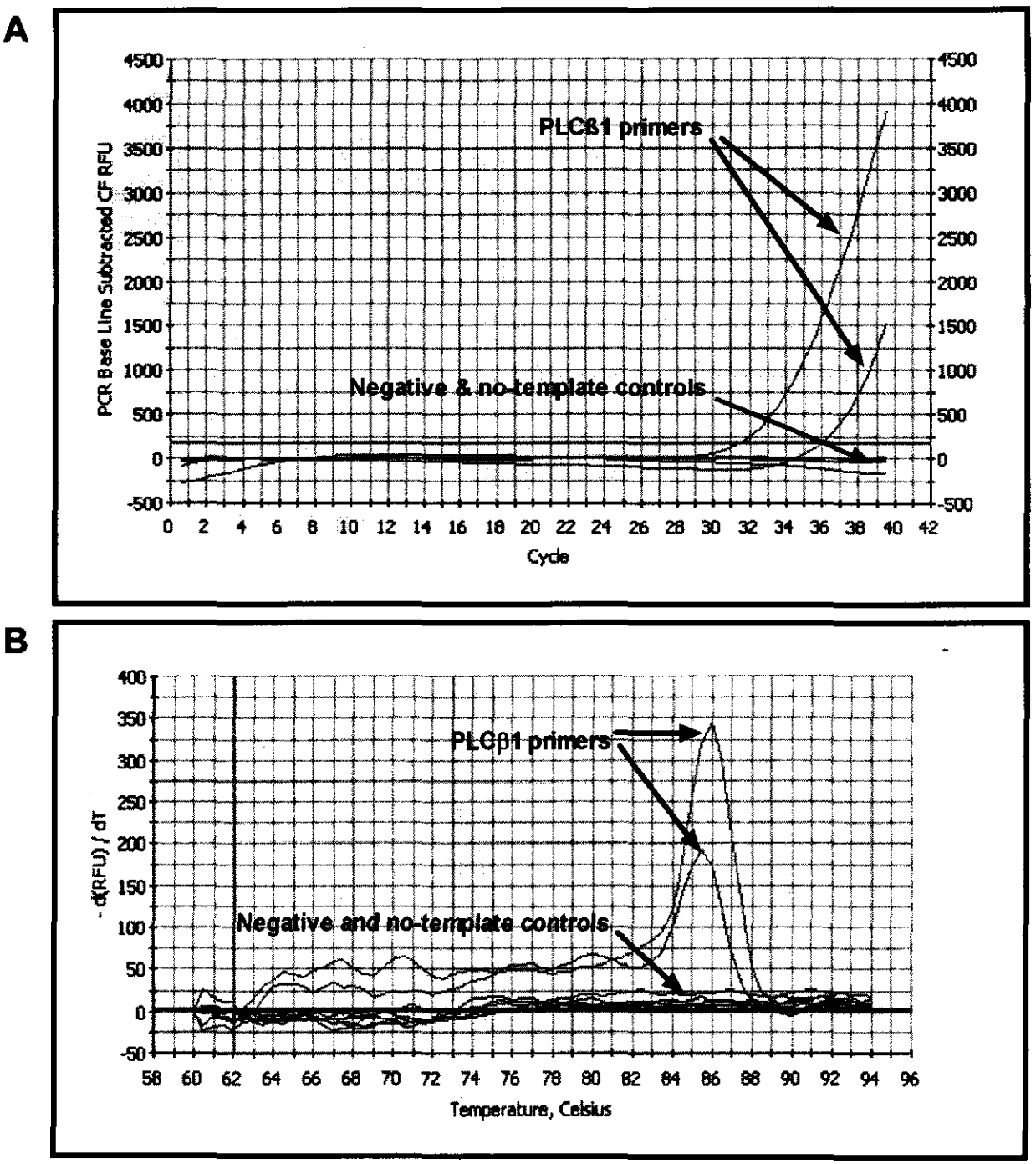


Figure B-13: *(A) Amplification curve of the cDNA for Phospholipase C β1 (PLCβ1) after various drug pre-treatments at conditions and temperatures as described in §3.5.1.3 (Experiment 1). (B) Melting curve of amplification product(s) with PLCβ1 primers after various drug pre-treatments (Experiment 1).*

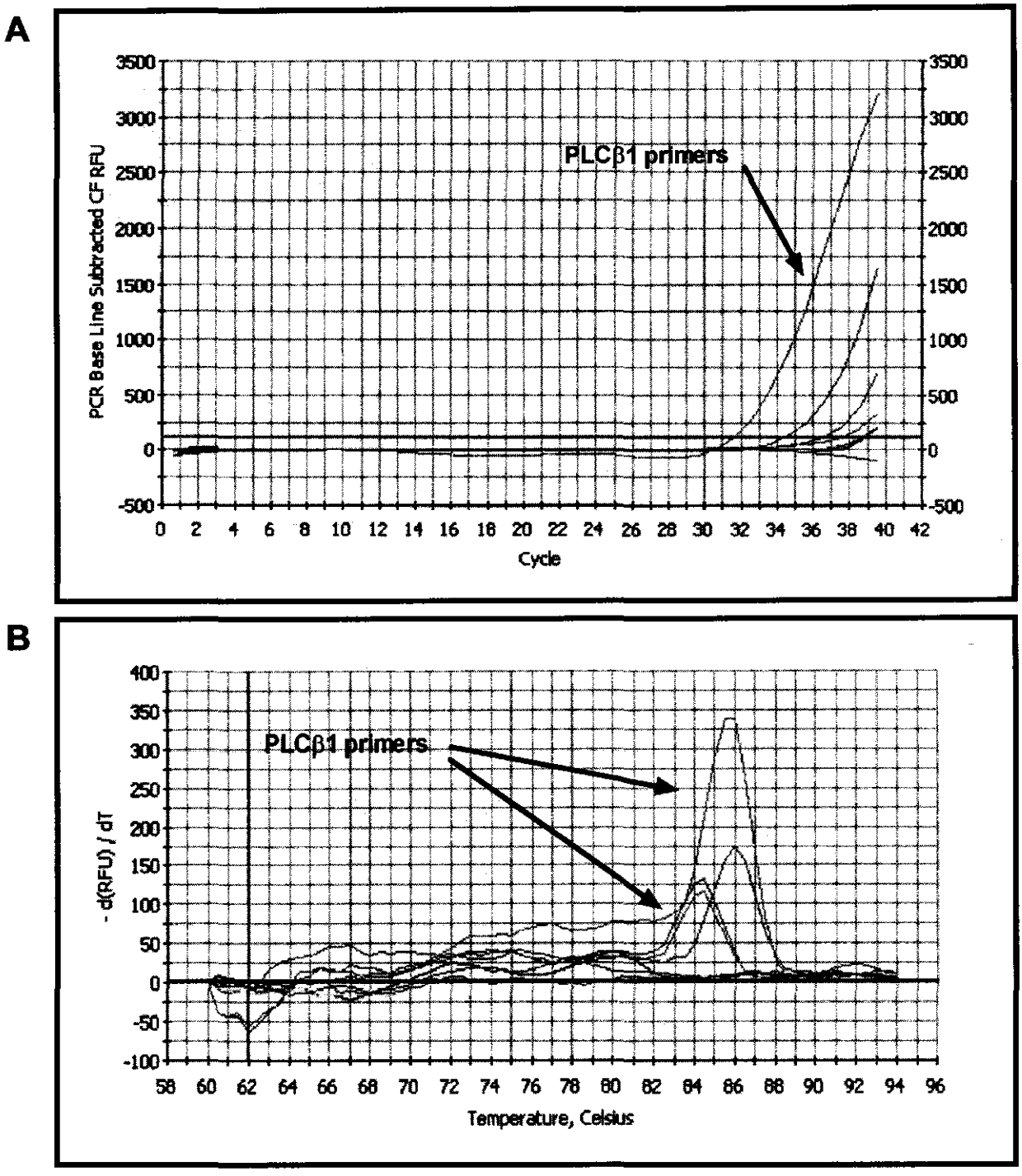


Figure B-14: *(A) Amplification curve of the cDNA for Phospholipase C β1 (PLCβ1) after various drug pre-treatments at conditions and temperatures as described in § 3.5.1.3 (Experiment 2). (B) Melting curve of amplification product(s) with PLCβ1 primers after various drug pre-treatments (Experiment 2).*

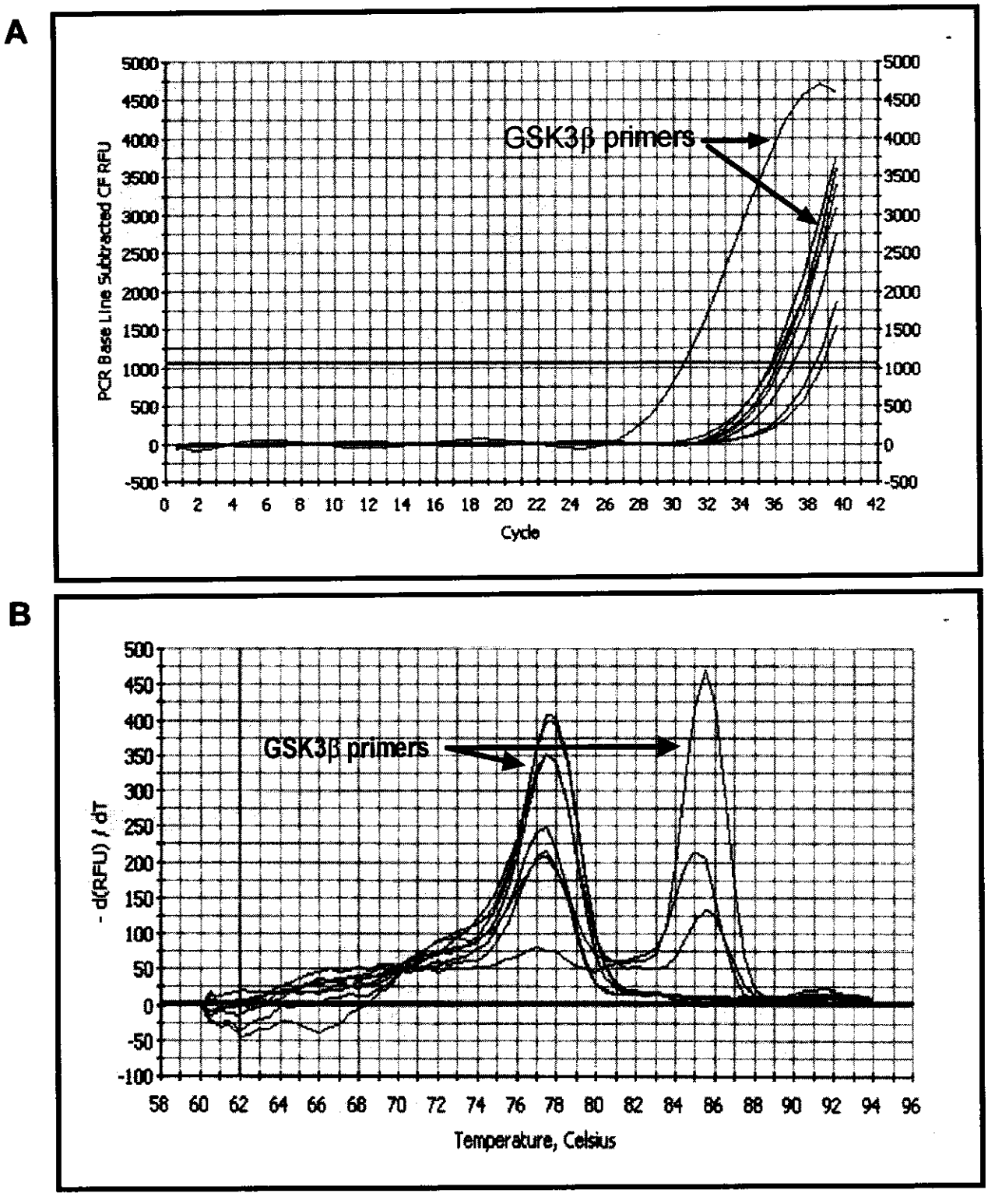


Figure B-15: *(A) Amplification curve of the cDNA for Glycogen synthase kinase 3β (GSK3β) after various drug pre-treatments at conditions and temperatures as described in §3.5.1.3 (Experiment 1). (B) Melting curve of amplification product(s) with GSK3β primers after various drug pre-treatments (Experiment 1).*

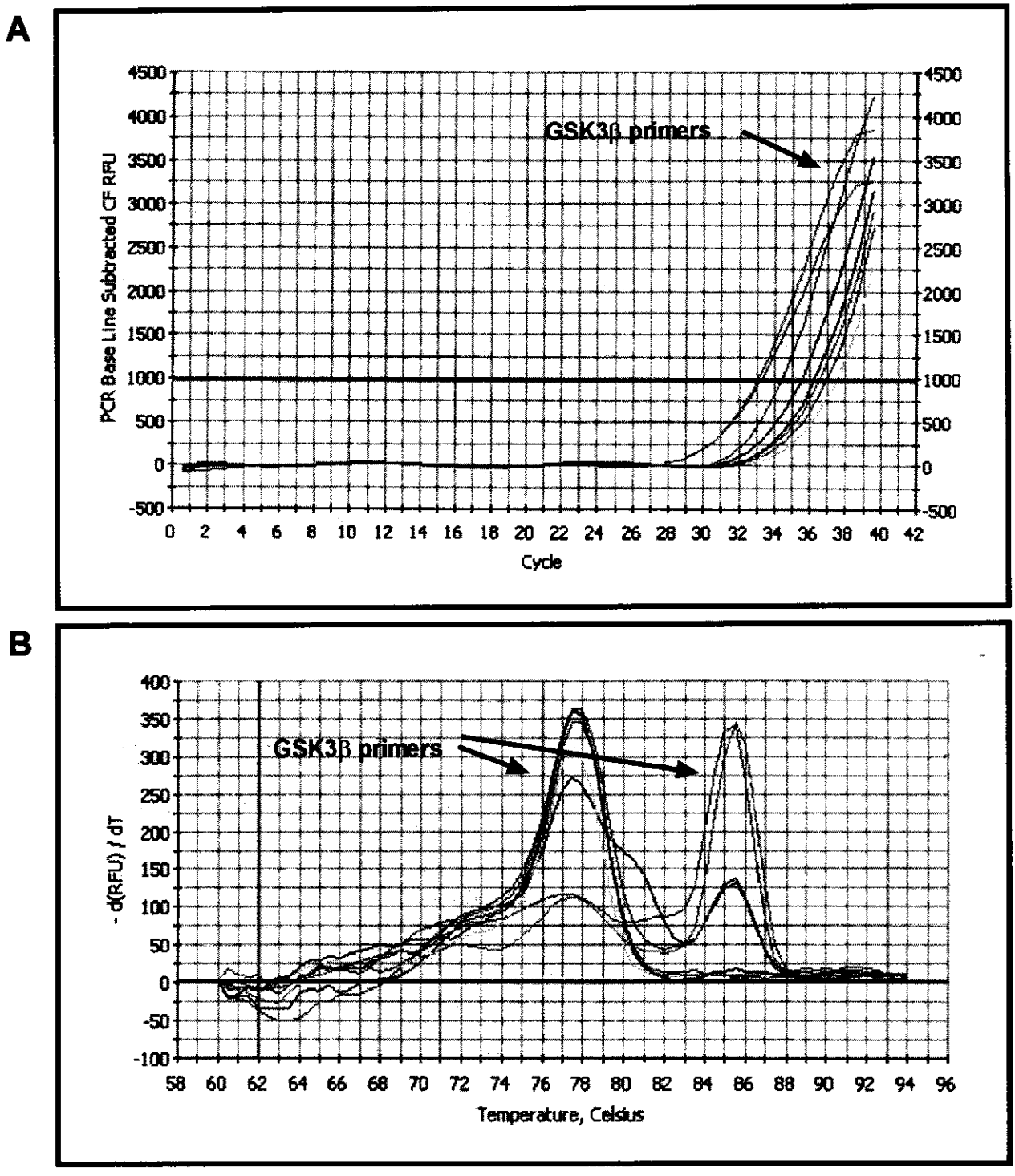


Figure B-16: *(A) Amplification curve of the cDNA for Glycogen synthase kinase 3β (GSK3β) after various drug pre-treatments at conditions and temperatures as described in §3.5.1.3 (Experiment 2). (B) Melting curve of amplification product(s) with GSK3β primers after various drug pre-treatments (Experiment 2).*

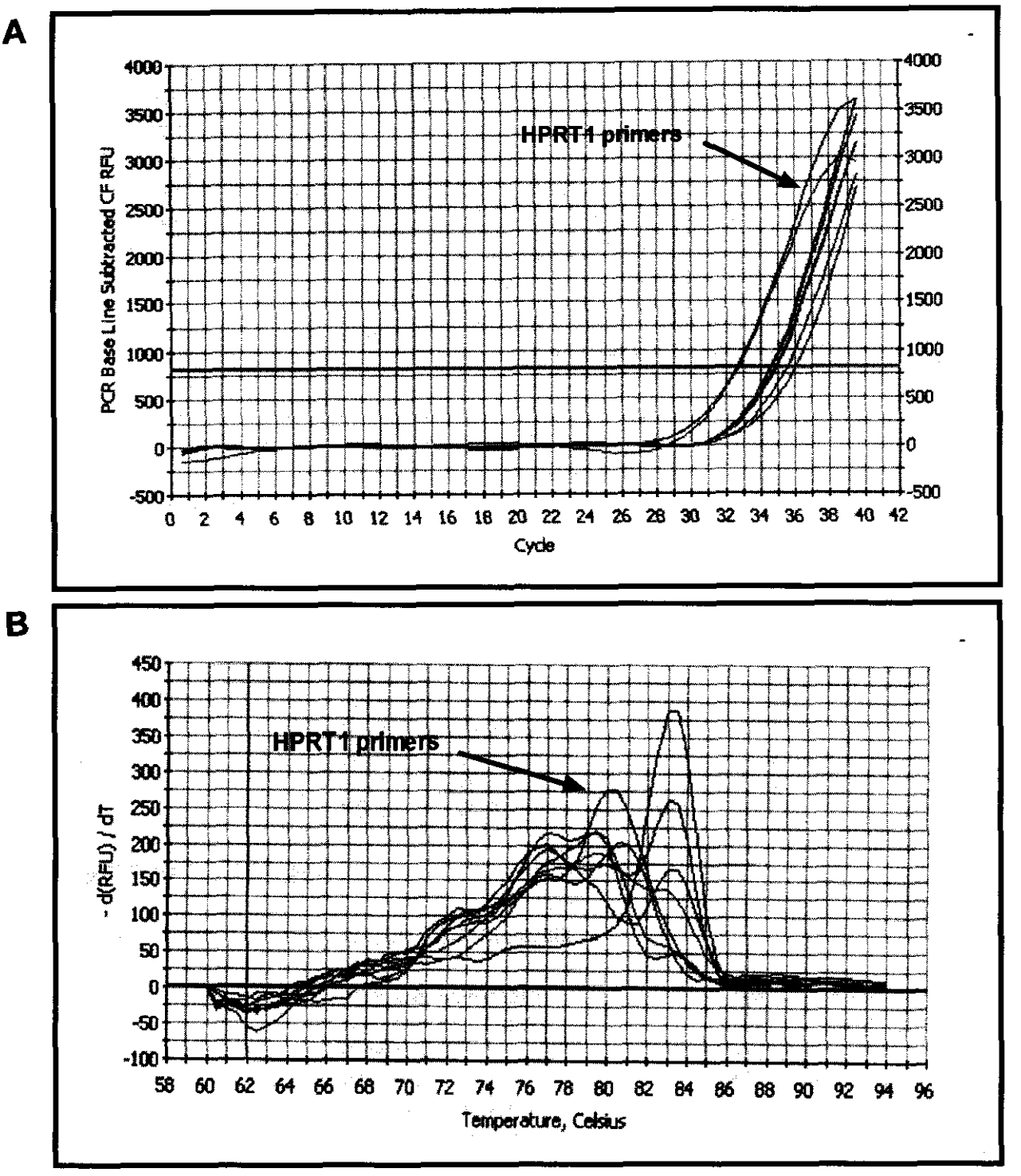


Figure B-17: *(A) Amplification curve of the cDNA for Hypoxanthine phosphoribosyl-transferase 1 (HPRT1) after various drug pre-treatments at conditions and temperatures as described in § 3.5.1.3 (Experiment 1). (B) Melting curve of amplification product(s) with HPRT1 primers after various drug pre-treatments (Experiment 1).*

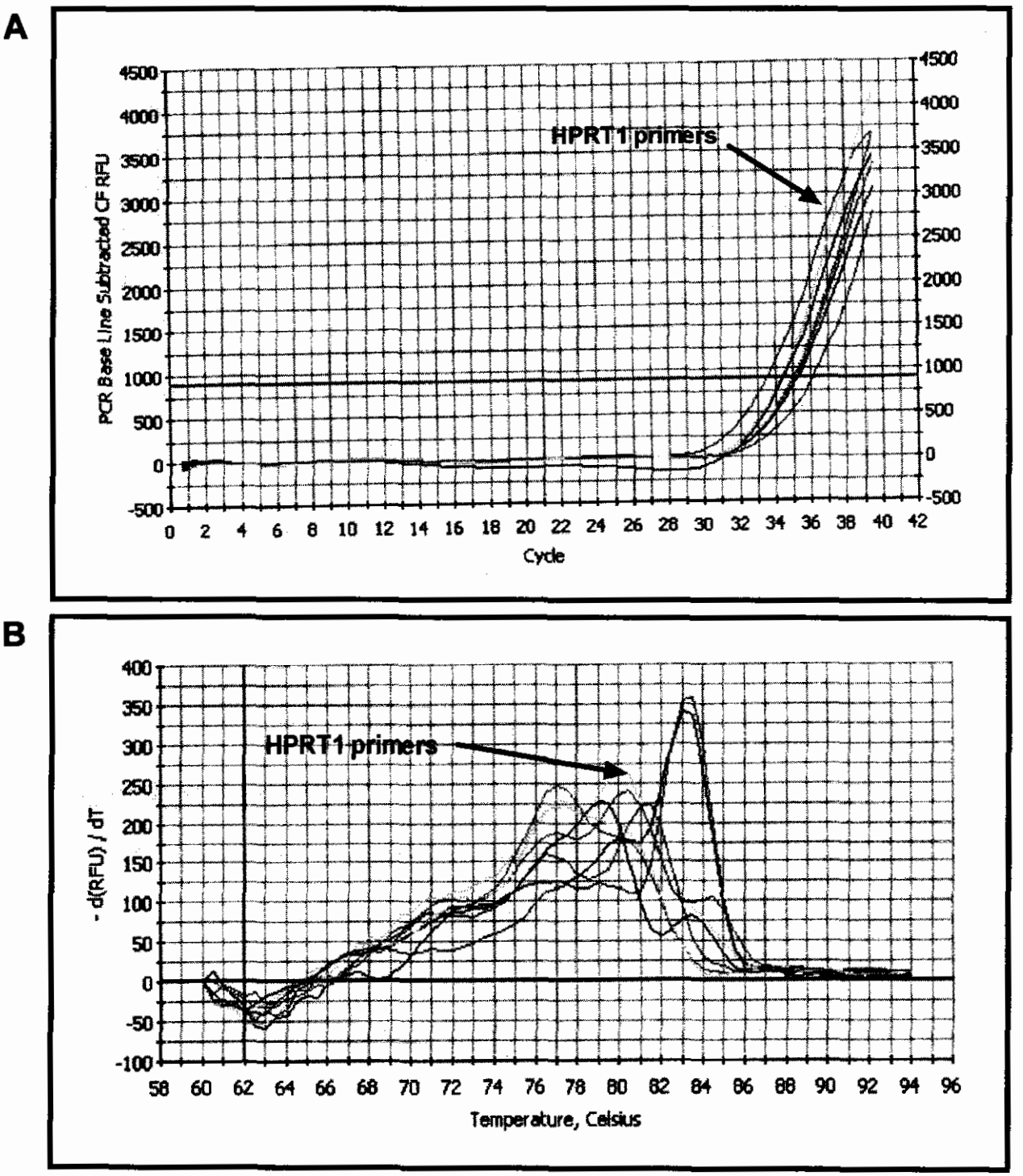


Figure B-18: *(A) Amplification curve of the cDNA for Hypoxanthine phosphoribosyl-transferase 1 (HPRT1) after various drug pre-treatments at conditions and temperatures as described in § 3.5.1.3 (Experiment 2). (B) Melting curve of amplification product(s) with HPRT1 primers after various drug pre-treatments (Experiment 2).*

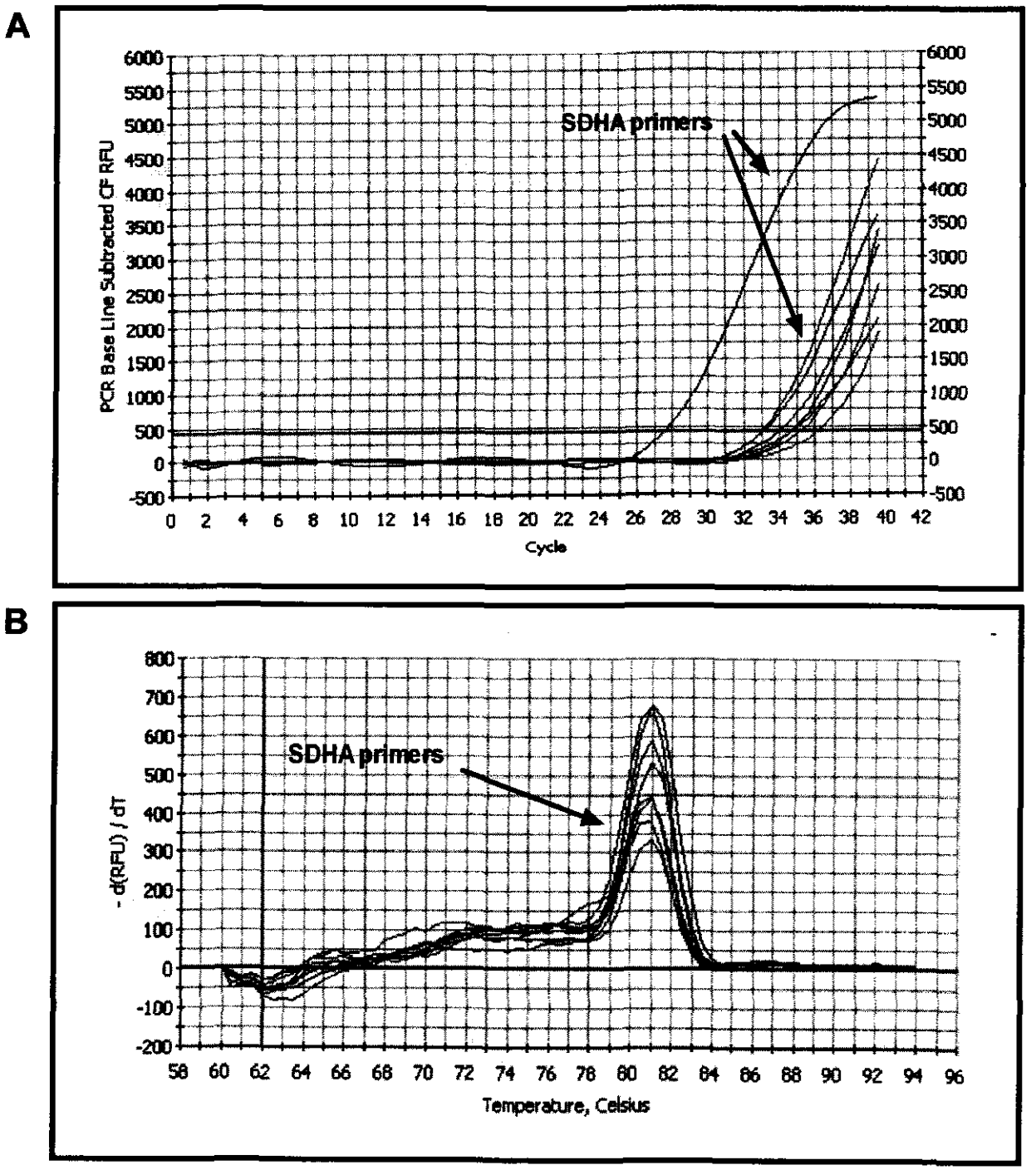


Figure B-19: *(A) Amplification curve of the cDNA for Succunate dehydrogenase complex, subunit A (SDHA) after various drug pre-treatments at conditions and temperatures as described in § 3.5.1.3 (Experiment 1). (B) Melting curve of amplification product(s) with SDHA primers after various drug pre-treatments (Experiment 1).*

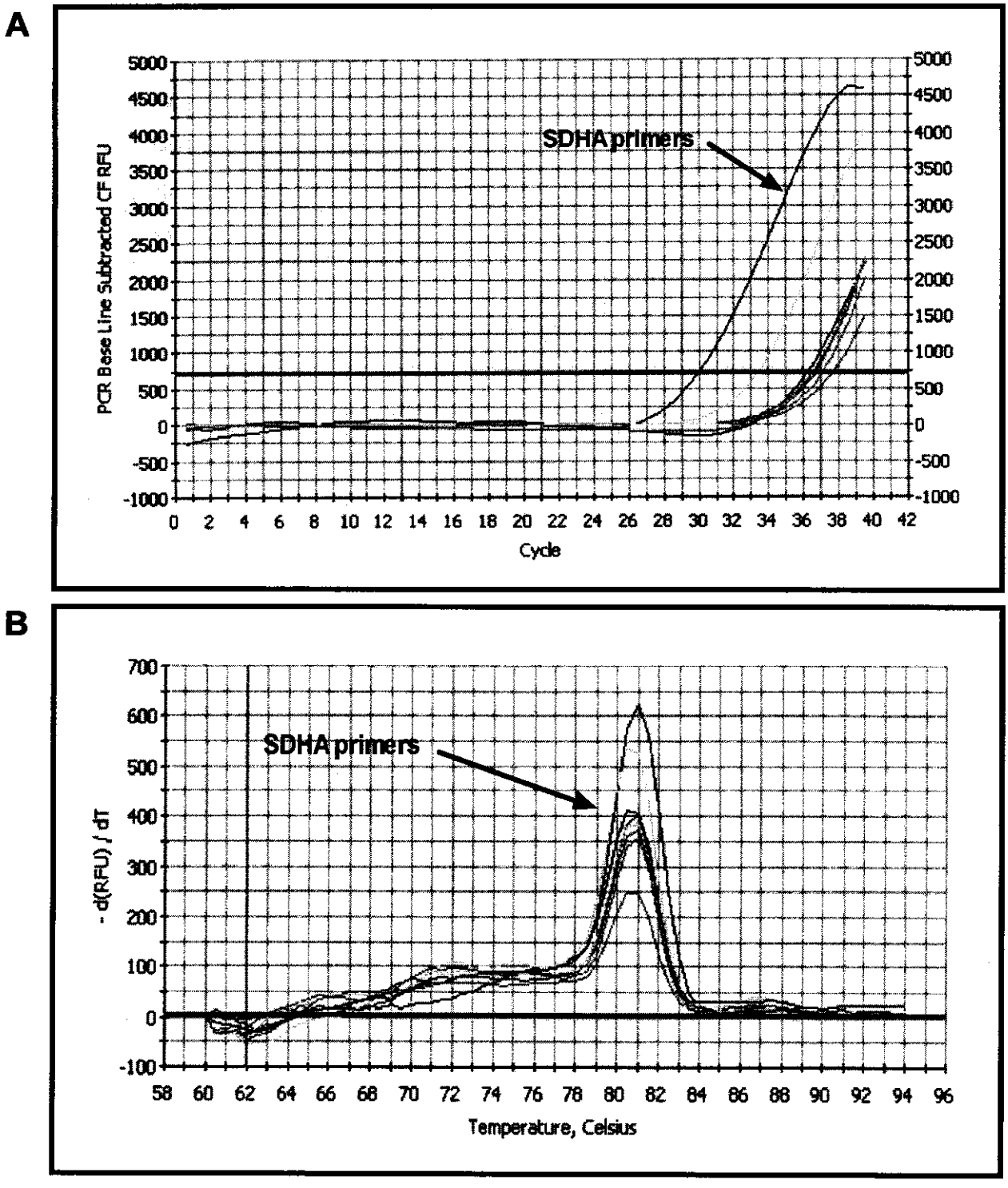


Figure B-20: *(A) Amplification curve of the cDNA for Succunate dehydrogenase complex, subunit A (SDHA) after various drug pre-treatments at conditions and temperatures as described in § 3.5.1.3 (Experiment 2). (B) Melting curve of amplification product(s) with SDHA primers after various drug pre-treatments (Experiment 2).*