cAMP-Dependent Protein Kinase Activity in Bipolar Affective Disorder

by

Melissa Albin

A thesis submitted in conformity with the requirements for the Degree of Master of Science, Institute of Medical Science, in the University of Toronto

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MSc Thesis- 2000
Melissa Albin
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ABSTRACT

Increasing evidence suggests that abnormal cAMP signaling contributes to the pathophysiology of bipolar affective disorder (BD). cAMP-dependent protein kinase (PKA), the primary downstream transducing enzyme of the cAMP system is involved in many cellular processes through the phosphorylation of substrate proteins. Reduced [³H]cAMP binding and increased PKA activity in postmortem brain from BD patients as compared with non-psychiatric controls (Rahman et al 1997; Fields et al 1999), support the notion that hyperfunctional cAMP signaling occurs in BD. The present study further examines the clinical relevance of PKA activity to BD in transformed living cells from affected individuals using a B lymphoblast cell line (BLCL) model that minimizes the influence of state-related factors. PKA activity was assayed using Kemptide as a substrate in the presence of a PKA-specific inhibitor.

Concentration-response relationships for cAMP-stimulated PKA activity were determined in BLCLs from BD patients (Bipolar I Disorder (BP-I), Bipolar II Disorder (BP-II)), major depressive disorder (MDD) patients and healthy subjects. There were no significant differences in basal or maximally-stimulated PKA activity nor in EC50 for cAMP activation of PKA activity between any of the patient groups and healthy subjects. A higher EC50 for cAMP activation of PKA activity was found in BLCLs from BP-II patients compared with BP-I patients showing a putative phenotype of elevated BLCL basal intracellular Ca²⁺ levels, relative to healthy subjects. Interestingly, BP-I patients with
lifetime comorbid diagnoses of anxiety disorders showed higher maximally-stimulated PKA activity than those with comorbid diagnoses of substance abuse. Additionally, age of onset of BD patients significantly correlated with maximally-stimulated PKA activity and BD patients with early onset (≤19 years) showed lower maximally-stimulated PKA activity than patients with adult onset (≥20 years). While differential PKA activity between BD patients and healthy subjects was not found, differences in PKA activity amongst BD patients stratified by various diagnostic characteristics may be relevant to the pathophysiology of BD.
ACKNOWLEDGEMENTS

I would like to dedicate this thesis to the memory of my grandparents, Betty and Sam Albin, and Irving Weiner.

I could not have completed this work without the continuous support, insight and guidance of Dr. Jerry Warsh. His constant availability and encouragement were very much appreciated, and were well beyond the call of duty.

I would like to thank Dr. Peter Li for all of his assistance. His insightful suggestions directed my learning process considerably and I thank him for all of his time and dedication to my work.

My work was also greatly supported by Dr. Catharine Whiteside for whom I am grateful for her advice and generous commitment of time.

This project would also not have been possible without the immense amount of support and guidance from Dr. Anat Fields, Marty Green, Dr. Arvind B. Kamble, David Sibony, Kin Po Siu and Cathy Spegg.

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ABBREVIATIONS

AC        adenyl cyclase
ADP       adenosine diphosphate
AKAP      cAMP-dependent protein kinase anchoring protein
AMPA      alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
5’AMP     5’-adenosine monophosphate
ATP       adenosine triphosphate
β-AR       β-adrenoceptor
BD        bipolar affective disorder
BDI       Beck Depression Inventory
BLCL      B lymphoblast cell line
BP-I      Bipolar I Disorder
BP-II     Bipolar II Disorder
C. elegans Caenorhabditis elegans
C-subunit catalytic subunit
cAMP      adenosine 3’ ,5’ monophosphate
Ca^{2+}   calcium ion
[Ca^{2+}]_B basal calcium concentration
CaM       calmodulin
CaMK      Ca^{2+}/calmodulin-dependent protein kinase
CaN       calcineurin
CAP       catabolite gene-activating protein
CARS-M    Clinician Administered Rating Scale for Mania
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<td>CAT</td>
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<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<tr>
<td>CBZ</td>
<td>carbamazepine</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 3',5' monophosphate</td>
</tr>
<tr>
<td>cGMPdk</td>
<td>cGMP dependent protein kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP responsive element modulator</td>
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<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
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<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
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<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
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<tr>
<td>DR</td>
<td>dopamine receptor</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>G protein</td>
<td>Guanine nucleotide-binding protein</td>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<td>GEF</td>
<td>guanine-nucleotide exchange factor</td>
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<td>GPCR</td>
<td>G protein coupled receptor</td>
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<td>GRK</td>
<td>G protein receptor kinase</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>HAM-D</td>
<td>Hamilton Depression</td>
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<td>5-HT</td>
<td>serotonin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
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<tr>
<td>IMPase</td>
<td>inositol monophosphatase</td>
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<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Li</td>
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<td>mAchR</td>
<td>muscarinic acetylcholine receptor</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>messenger ribonucleic acid</td>
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<td>NES</td>
<td>nuclear export signal</td>
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<td>nuclear pore complex</td>
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<td>phosphoenolpyruvate carboxykinase</td>
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<tr>
<td>PI</td>
<td>phosphoinositide</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>PKI</td>
<td>PKA heat-stable inhibitor</td>
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<td>PLC</td>
<td>phospholipase C</td>
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PP: protein phosphatase
PP-2B: calcineurin
PSP: protein serine/threonine phosphatase
R-subunit: regulatory subunit
RED: Repeat Expansion Detection
RT: room temperature
SCID-NP: Structured Clinical Interview for DSM-IV Axis I Disorders-Non-Patient
SD: standard deviation
VPA: valproate
CHAPTER I
OVERVIEW
I.1 Introduction

Bipolar affective disorder (BD) comprises a group of serious, episodic illnesses that involve broad and disabling changes in mood, thinking, energy, and actions. The spectrum of illnesses that it encompasses has been subdivided into operationally defined subtypes based primarily upon phenomenological characteristics (Andreason 1986; Goodwin & Jamison, 1990; Akiskal 1996). BD is a relatively common disorder (lifetime prevalence of 1.2%), with significant morbidity, mortality (suicide rates of 20%), and comorbidity with alcohol and drug abuse or dependence (alcohol abuse and alcoholism rates of 35%, drug abuse rates of 41%) (Goodwin & Jamison 1990; Dilsaver et al 1994). While currently available pharmacological treatments promote recovery and prevent relapse, a substantial proportion of patients responds incompletely or not at all (Keck Jr & McElroy 1996; Manji et al 1996). Furthermore, the mechanism of action for the effectiveness of existing pharmacological treatments is not fully known (Jope 1999a, b).

The etiological and pathophysiological bases for BD are poorly understood (Warsh & Li 1996; Li et al 2000; Warsh et al., 2000). This may be attributed to the purported heterogeneity of the disorder (Goodwin & Jamison 1990; DePaulo Jr & McMahon 1996; Potash & DePaulo Jr 2000). Support for the role of biological factors in the pathophysiology of BD comes from family (Winokur et al 1982), twin (Kendler et al 1993; Cardno et al 1999) and genetic studies (De Paulo Jr & McMahon 1996; NIMH Genetics Initiative Bipolar Group 1997), findings of neurobiochemical abnormalities in BD patients (Warsh & Li 1996; Li et al 2000), and studies examining the biochemical and molecular effects of mood-stabilizing agents (Manji et al 1996; Jope 1999a, b). One potential target
for neurobiochemical abnormalities in BD patients is cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), a critical intracellular transducing protein in the cAMP-mediated signaling cascade and the focus of this thesis (Beebe 1994; Francis & Corbin 1994; Francis & Corbin 1999).

BD is currently subcategorized into three main subtypes based on duration, severity and presence or absence of psychosis and include Bipolar I (BP-I), Bipolar II (BP-II), and Cyclothymic Disorder. BP-I is characterized by the occurrence of one or more manic or mixed episodes and recurrent major depressive episodes, although unipolar mania has been described as well. BP-II is characterized by one or more major depressive episodes accompanied by at least one hypomanic episode. Manic episodes are distinguished from hypomanic episodes based on severity and duration: Manic episodes are at least 1 week of sustained, abnormal, expansive or irritable mood, while hypomanic episodes may be of shorter duration (at least 4 days). Pathological moods are accompanied by disturbances in thinking, psychomotor activity, and circadian rhythms. Hypomanic episodes are further distinguished from mania by the absence of psychotic symptoms and less severe impairment in social and/or occupational functioning. Major depressive episodes are defined as at least 2 weeks of depressed mood or loss of interest or pleasure in nearly all activities. The episodes are also accompanied by disturbances in thinking, psychomotor activity, and circadian rhythms. A mixed episode is at least 1 week, during which criteria are met for both manic and major depressive episodes nearly every day. Finally, Cyclothymic Disorder is characterized by at least 2 years of periodic hypomanic and depressive symptoms which do not meet the criteria for manic or major depressive episodes, respectively (DSM-IV, 1994).
Recent studies focused on identifying genes that contribute to the vulnerability of developing BD support the view that these illnesses display complex (non-Mendelian) patterns of inheritance indicative of complex trait disorders (Berrettini 1994; Stine et al 1995; NIMH Genetics Initiative Bipolar Group 1997). Twin studies have reported high estimates of familial comorbidity (44.0% in monozygotic twins and 9.1% in dizygotic twins for BP-I and BP-II and combined) (Cardno et al 1999). Family studies have demonstrated increased rates of BD and other affective disorders in the relatives of BD patients as compared with the relatives of healthy subjects (Winokur et al 1982). Adoption studies show greater psychopathology in the biological parents of BD patients compared to the adoptive parents (Mendlewicz & Rainer 1977). While the estimates of heritability for the disorder are consistent (79% [Kendler et al 1993] to 87% [Cardno et al 1999] for BP-I and BP-II), the modes of inheritance are complicated by its heterogeneity and have yet to be established (De Paulo Jr & McMahon 1996).

Early studies searching for biochemical abnormalities in BD patients were heavily influenced by the observations that antidepressant medications modified neurotransmitter turnover and receptor function. The studies focused on the levels, turnover and functionality of various neurotransmitters and their receptors in the central nervous system (CNS) in attempts to clarify the pathophysiological basis of the illness (Extein et al 1979; Pandey et al 1979; Warsh et al 1988). The original hypotheses, however, did not sufficiently explain the disorder as there were inconsistent correlates with changes in the levels and activity of receptors or neurotransmitters and active states of illness (Jeanningros et al 1991). Furthermore, the action of mood stabilizing agents could not be adequately explained by effects on neurotransmitter turnover and receptor sensitivity alone, since these
were not necessarily affected at therapeutically relevant concentrations of these agents. This prompted the search for abnormalities beyond the level of first messengers and receptors to the level of various second messengers and their signal transduction pathways (Warsh et al 1988; Wachtel 1990; Hudson et al 1993; Manji et al 1995a; Warsh & Li 1996).

Second messenger mediated signal transduction pathways, which link external stimuli acting on receptors to intracellular events, are now being recognized as complex cascades that are still poorly understood (Berridge 1993; Daniel et al 1998; Smith et al 1999). cAMP, calcium (Ca^{2+}), and the phosphoinositide-(PI) derived second messengers, inositol triphosphate (IP_{3}) and diacylglycerol (DAG) are prominent intracellular signaling molecules involved in many aspects of cellular signaling (Smith et al 1999). The recent expansion of knowledge of the molecular pathways of second messengers and the evidence of abnormalities of these messengers in BD and other mood disorders have directed the focus of research on the pathophysiology of BD to the signal transduction pathways mediated by these second messengers, as well as the interactions between the pathways (Warsh & Li 1996; Li et al., 2000; Warsh et al., 2000). Integral components of the signal transduction pathways are protein kinases, a large family of enzymes that regulate a myriad of cellular processes through the phosphorylation of cellular proteins (Girault 1993; Beebe 1994; Smith et al 1999).

In the following sections, current understanding of the cAMP-mediated signal transduction system as it funnels through PKA regulation and action is discussed, as well as some of the important crosstalk interactions that occur with and between other transduction systems which also may be disturbed in BD. Evidence implicating disturbances of these
systems in the pathophysiology of BD and the effects of various mood-stabilizing agents on
the systems are presented. This is followed by a description of the objectives and the
research strategy that was undertaken to address the specific hypotheses of this thesis.

1.2 cAMP-Mediated Signal Transduction Cascade

The cAMP-mediated signal transduction cascade, depicted in Figure 1, is initiated
by the binding of ligands to specific subtypes of the serpentine receptor superfamily of
guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) that couple to
the stimulatory or inhibitory heterotrimeric G proteins. These G proteins, when activated,
in turn stimulate or inhibit the effector adenylyl cyclase (AC), which catalyzes the
conversion of adenosine triphosphate (ATP) to cAMP. The cAMP thus formed diffuses
throughout the cytosol to modulate PKA activity and its subsequent phosphorylation of
numerous proteins that are involved in a wide range of cellular processes (Beebe 1994).

1.2.1 Guanine Nucleotide-Binding Protein Coupled Receptors

Agonists, such as the neurotransmitters norepinephrine, dopamine and
acetylcholine, induce conformational changes in specific receptors, including β-adrenergic
(β-AR), dopaminergic (DR) and muscarinic acetylcholinergic receptors (mAchR)
(reviewed in Lefkowitz et al 1983; Gudermann et al 1996; Zastawny et al 1997). These
GPCRs are transmembrane proteins consisting of an extracellular amino terminus, seven
stretches of hydrophobic amino acid domains that form transmembrane α helices joined by
alternating extracellular and intracellular loops, and an intracellular carboxyl terminus
(Strader et al 1995; Gudermann et al 1996; Zastawny et al 1997). Agonists acting on
GPCRs bind to specific sites within hydrophilic regions of the transmembrane domains and
Neurotransmitter or Hormone
"First Messengers"

\[
\begin{align*}
\text{Receptor} & \quad \text{Adenylyl Cyclase} \\
G \text{ Protein} & \quad \text{Phosphodiesterase} \\
\text{ATP} & \quad \text{cAMP} \quad \text{"Second Messengers"} \\
\text{cAMP} & \quad \text{cAMP-Dependent Protein Kinase} \\
\text{Inactive} & \quad \text{Activated}
\end{align*}
\]

Figure 1: cAMP-mediated signal transduction cascade
activate the receptors (Dickey et al 1987; Zastawny et al 1997). The agonist-liganded receptor exists as a transient complex that interacts with the G proteins promoting their binding with guanosine triphosphate (GTP) and dissociation into an α-subunit and a βγ-dimer that interact with effector enzymes in the plane of the membrane to initiate cascades of signaling events (Maguire et al 1976; Lefkowitz et al 1983; Dickey et al 1987; Gilman 1987).

Advances made over the past decade in understanding the structure and regulation of the cAMP-mediated signal transduction pathway indicate a complex array of signal modulating processes that occur through the receptor-G protein-effector apparatus (Gilman 1987; Gudermann et al 1996; Zastawny et al 1997). Inputs from the first messenger level are amplified or attenuated, "multiplexed" into a number of intracellular signaling cascades in some instances or channeled into a single second messenger system in others. Multiple types of GPCRs can "converge" on the same and/or different G protein(s) achieving signal convergence or divergence. To allow for specificity for these different functions within the family of GPCRs there are a number of receptor subtypes that possess different signal transduction properties and tissue expression (Zastawny et al 1997). It is important to note that there are constraints to the receptor-G protein interactions due to different compartmentalization and affinities between the constituents. In turn, it is postulated that upon ligand binding a single receptor may achieve different conformations that will preferentially couple to distinct G proteins (Gudermann et al 1996). In addition, amplification of extracellular signals is accomplished by GPCRs through coupling to many G proteins upon activation (Nurnberg et al 1995).
**I.2.2 Guanine Nucleotide-Binding Proteins**

G proteins comprise a large family of GTP-binding proteins or GTPases that are activated by GPCRs and thus involved in signal transduction (Gilman 1987; Bourne et al 1990; Nurnberg et al 1995). Heterotrimeric G proteins are composed of α- (39 – 52 kDa), β- (35-44 kDa), and γ- (6 – 8 kDa) subunits, and are classified by their α-subunit composition (reviewed in Simon et al 1991; Watson et al 1996). Based upon the degree of homology in amino acid sequence and functionality, the 23 known α-subunits have been subdivided into four main families: Gs, Gi, Gq, and Gi2 (See Table 1 for details) (reviewed in Gudermann et al 1996; Exton 1997). GTPase activity is intrinsic to the α-subunits (Fung 1983) which are known to regulate various effectors such as AC in signal transduction cascades (Lefkowitz et al 1983; Gilman 1987; Clapham & Neer 1993; Nurnberg et al 1995). There are 5 known β-subunits and 12 known γ-subunits that have been cloned and their amino acid sequences deduced. The β- and γ-subunits form a tight, noncovalent bound dimer (Morishita et al 1995; Nurnberg et al 1995; Gudermann et al 1996). There is now substantial evidence showing that βγ-dimers, previously thought to function only as membrane anchors, also participate directly in G protein-mediated activation of effectors (Clapham & Neer 1993), and may play a significant role in desensitization of GPCRs (Zastawny et al 1997).

In their inactive state heterotrimeric G proteins exist in trimeric (αβγ) form with guanosine diphosphate (GDP) bound to the α subunit. The trimeric protein interacts with distinct cytoplasmic segments of an agonist-activated GPCR forming a short-lived “high-affinity ternary complex” which promotes the dissociation of GDP and binding of GTP
### Table 1-Mammalian Gα Subunits

<table>
<thead>
<tr>
<th>α subunit</th>
<th>Effector</th>
<th>Tissue Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gs</td>
<td>↑ AC, Ca(^{2+}) channel</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Golf</td>
<td>↑ AC</td>
<td>Olfactory epithelium &gt; others</td>
</tr>
<tr>
<td>Gt1</td>
<td>↑ cGMP-phosphodiesterase</td>
<td>Rod photoreceptors</td>
</tr>
<tr>
<td>Gt2</td>
<td>↑ cGMP-phosphodiesterase</td>
<td>Cone photoreceptors</td>
</tr>
<tr>
<td>Ggust</td>
<td>↑ AC, ↑ K(^{+}) channel</td>
<td>Taste cells</td>
</tr>
<tr>
<td>Gil</td>
<td>↓ AC, ↑ K(^{+}) channel</td>
<td>Neural &gt; other</td>
</tr>
<tr>
<td>Gi2</td>
<td>↓ AC, ↑ K(^{+}) channel</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Gi3</td>
<td>↓ AC, ↑ K(^{+}) channel</td>
<td>Other &gt; neural</td>
</tr>
<tr>
<td>Go</td>
<td>↓ Ca(^{2+}) channel</td>
<td>Neural, endocrine</td>
</tr>
<tr>
<td>Gz</td>
<td>↓ AC</td>
<td>Neural, platelets</td>
</tr>
<tr>
<td>Gq</td>
<td>↑ PLCβ1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>G11</td>
<td>↑ PLCβ1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>G14</td>
<td>↑ PLCβ1</td>
<td>Liver, lung, kidney</td>
</tr>
<tr>
<td>G15/16</td>
<td>↑ PLCβ1</td>
<td>Blood cells</td>
</tr>
<tr>
<td>G12</td>
<td>Na(^{+})/H(^{+}) exchanger</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>G13</td>
<td>Na(^{+})/H(^{+}) exchanger</td>
<td>Ubiquitous</td>
</tr>
</tbody>
</table>

The mammalian Gα subunits are divided into four main families based upon amino acid sequence and effector regulation similarities. ↓ indicates a decrease in activity while ↑ indicates an increase in activity.
with high-affinity to the α-subunit (Maguire et al 1976; Lefkowitz et al 1983; Dickey et al 1987). The binding of GTP activates a switch mechanism that results in a conformational change in the G protein (Fung 1983) and dissociation of the G protein from the receptor. The receptor then reverts to a low-affinity conformation and the agonist dissociates from the receptor (Lefkowitz et al 1983). The trimeric G protein dissociates into a GTP-bound α-subunit and a βγ-dimer (Bourne et al 1990; Nurnberg et al 1995). Both the dissociated GTP-bound α-subunit and the βγ-dimers are then free to interact with specific effectors in the cell (Clapham & Neer 1993; Beebe 1994; Gudermann et al 1996). The intrinsic GTPase activity of the α-subunit terminates the cycle by hydrolyzing GTP to GDP, which results in the release of inorganic phosphate and allows the reassociation of the GDP-bound α-subunit with the βγ-dimer thereby reforming the inactive G protein (Bourne et al 1990).

The different G protein subunits participate in diverse processes. The interactions of the G protein subunits with various effectors allow for signal amplification or attenuation, divergence and convergence of signals, and coincidence detection (Gilman 1987; Clapham & Neer 1993; Iyengar 1993). (See Table 1 for details). For example, the activated α-subunits and βγ-dimers can give rise to bifurcating signals. The Gia-subunit inhibits AC while the activated Giβγ-dimers stimulate phospholipase C (PLC) which is involved in Ca²⁺-mediated signaling (Clapham & Neer 1993; Gudermann et al 1996).
1.2.3 Adenylyl Cyclase

One of the major effector enzymes regulated by G proteins is the membrane-bound AC. Nine subtypes of mammalian AC have been identified, each with distinct regulatory properties, as well as unique cell-type and tissue distributions (see Table 2) (reviewed in Tang & Hurley 1998; Taussig & Zimmerman 1998). The basic structure of the enzyme is two cytoplasmic domains (C₁₁(C₁₆ and C₁₉) and C₂₂(C₂₆ and C₂₈)), each of which follows a transmembrane stretch. The two cytoplasmic domains form a "wreath-like" dimer arrangement that creates an interface providing sites for binding and catalysis. The GTP-bound α-subunit of the G protein binds to the amino terminus of the C₁₆ region and the hydrophobic groove of the C₂₆ region causing AC to undergo conformational changes (Tang & Hurley 1998; Taussig & Zimmerman 1998). The substrate for AC, ATP, will then bind to a hydrophobic pocket created by the cytoplasmic domain interface thereby inducing further conformational changes in AC. AC in turn catalyzes the conversion of ATP to cAMP (Tang & Hurley 1998). The newly generated cAMP is then available for binding to specific sites on PKA, the main focal point for the transduction of the cAMP signal (Beebe 1994), and activating the enzyme.

The variety of regulatory inputs modulating the function of the different AC subtypes (summarized in Table 2) exemplify the roles of this family of effectors in the integration of signals from a variety of sources, adding to the complexity of the cAMP-dependent signal transduction pathway (Sunahara et al 1996; Taussig & Zimmerman 1998). A common characteristic of all AC subtypes is that they are stimulated by Gsα. The effectors allow for the amplification and attenuation of signaling, and are also
Table 2-Regulation of mammalian Adenylyl Cyclases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ca(^{2+})</th>
<th>βγ</th>
<th>α₁</th>
<th>α₀</th>
<th>α₂</th>
<th>PKA</th>
<th>PKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>▼</td>
<td>?</td>
<td>↑</td>
</tr>
<tr>
<td>II</td>
<td>nc</td>
<td>↑(+α₄)</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>?</td>
<td>↑</td>
</tr>
<tr>
<td>III</td>
<td>↑(+α₄)</td>
<td>nc</td>
<td>?</td>
<td>nc</td>
<td>nc</td>
<td>?</td>
<td>↑</td>
</tr>
<tr>
<td>IV</td>
<td>nc</td>
<td>↑(+α₄)</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>?</td>
<td>nc/▼</td>
</tr>
<tr>
<td>V</td>
<td>▼</td>
<td>nc</td>
<td>▼</td>
<td>nc</td>
<td>▼</td>
<td>▼</td>
<td>nc/▼</td>
</tr>
<tr>
<td>VI</td>
<td>▼</td>
<td>nc</td>
<td>▼</td>
<td>nc</td>
<td>▼</td>
<td>▼</td>
<td>nc/▼</td>
</tr>
<tr>
<td>VII</td>
<td>nc</td>
<td>↑</td>
<td>?</td>
<td>nc</td>
<td>nc</td>
<td>?</td>
<td>▼</td>
</tr>
<tr>
<td>VIII</td>
<td>↑</td>
<td>↓</td>
<td>▼</td>
<td>nc</td>
<td>nc</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>IX</td>
<td>▼</td>
<td>nc</td>
<td>?</td>
<td>nc</td>
<td>nc</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

The large, multigene family of adenylyl cyclases (AC) are made up of nine distinct subtypes which are all stimulated by Gsα. The subtypes are differentially regulated by Ca\(^{2+}\), G protein βγ subunits, Gα subunits, Goα subunits, Gzα subunits, PKA and PKC. ↑ indicates a significant increase in activity; ▼ indicates a significant decrease in activity; nc indicates no change in activity; and +Gsα indicates synergism with the Gsα.
important points for crosstalk between various transduction systems (Sunahara et al 1996). For example, AC type II has been shown to integrate signals from Gs, Gi and Gq allowing for the enhancement of extracellular signals (Lustig et al 1993). Furthermore, the AC subtypes are differentially expressed in various mammalian tissues and cell-types adding to the specificity of signaling (Sunahara et al 1996; Taussig & Zimmermann 1998).

I.3 cAMP-Dependent Protein Kinase

The biologically relevant effects of cAMP are mostly mediated by the activation of PKA (Taylor et al 1990; Beebe 1994; Francis & Corbin 1994; Francis & Corbin 1999; Smith et al 1999) which was discovered by Walsh et al (1968). In the absence of cAMP, the PKA holoenzyme exists as an inactive tetramer composed of two catalytic (C)-subunits bound to a regulatory (R)-subunit dimer (reviewed in Taylor et al 1990; Spaulding 1993; Beebe 1994; Francis & Corbin 1994; Tasken et al 1997). On the basis of their elution patterns by anion exchange column chromatography, two different PKA isozymes (PKA-I and PKA-II) were identified, which were shown to be comprised of two different R-subunits, RI and RII (43 and 45 kDa, respectively) (Taylor et al 1990; Francis & Corbin 1999; Smith et al 1999). Each R-subunit has two binding sites for cAMP, and upon binding of cAMP the holoenzyme dissociates into an R$_2$(cAMP)$_4$ dimer and two catalytically active C-subunits. The C-subunits are then free to phosphorylate substrate proteins through the catalytic transfer of the γ-phosphate of ATP to Ser or Thr residues in consensus sequences of substrate proteins (reviewed in Hansson et al 1999). Protein phosphorylation by PKA is known to regulate a number of cellular processes including gene transcription (Nestler & Greengard 1983; Girault 1993).
I.3.1 Regulatory Subunit Subtypes

The two major R-subunits identified, RI and RII, can be further subdivided, based on the identification of separate gene products, into four subtypes: RIα (Lee et al 1983), RIβ (Clegg et al 1988), RIIα (Scott et al 1987), and RIIβ (Jahnsen et al 1986). RIα and RIβ subunits show a high degree of homology at both the nucleotide and amino acid level (Clegg et al 1988), as do the RIIα and RIIβ subunits (Tasken et al 1997). Further examination of the RIα gene has revealed the existence of two alternately spliced RIα mRNAs (RIα1a and RIα1b) resulting from two different leader exons controlled by distinct promoters (Solberg et al 1997). The R-subunits are most divergent at the amino-terminal portions of the proteins highlighting potential regions for possible differential regulation of activity and expression (Clegg et al 1988), perhaps through the association with different binding proteins (Colledge & Scott 1999).

I.3.2 Catalytic Subunit Subtypes

Three distinct C-subunits have been identified through molecular cloning, termed Cα (Uhler et al 1986a), Cβ (Uhler et al 1986b) and Cγ, which has only been detected in human testis (Beebe et al 1990). An inactive Cα splice variant, Cα2, is truncated at the C-terminal region (Thomis et al 1992). Two alternative splice variants of the murine Cβ1 gene, Cβ2 and Cβ3, which arise from the initiation of transcription by different promoters, have been isolated and characterized (Guthrie et al 1997). A splice variant similar to the murine Cβ2 was found for the bovine Cβ gene (Wiemann et al 1991), and it has been suggested that the differences in the amino-terminal regions of the Cβ splice variants might
have important functional consequences (Guthrie et al 1997). Very recently, at least 12 different C-subunit transcripts generated through alternative splicing of a single RNA were reported in *Caenorhabditis elegans* (*C. elegans*), further highlighting the complex structural diversity of the C-subunits (Tabish et al 1999).

In total, 24 different holoenzymes have been described that can possibly be expressed in mammalian tissues with homologous R-subunits forming dimers within the holoenzymes (Francis & Corbin 1994). R1α-R1β heterodimers have been demonstrated *in vivo* and *in vitro*, however, thereby increasing the number of possible different types of isozymes (Tasken et al 1993).

I.3.3 Domain Structure of Regulatory Subunits

The R-subunits, the major receptor for cAMP in eukaryotic cells (Taylor et al 1990), contain an amino-terminal dimerization domain, an inhibitory region, or the "hinge" region, which interacts with the C-subunits of the holoenzyme, and two tandem cAMP binding sites in the carboxyl terminus (see Figure 2) (Leon et al 1997; reviewed in Francis & Corbin 1999). The deduced amino acid sequences of the dimerization domains are most divergent within the family of R-subunits, suggesting isoform-specific functions for the dimerization domains (Clegg et al 1988). The dimerization domain corresponds to the first 62 amino acids in RI and the first 45 amino acids in RII (Huang et al 1997). Examination of the R1α dimerization domain shows that the region has a strong α-helical content. The two subunits of the R1α dimer are linked by interchain disulfide bonds. The disulfide
Figure 2: Domain structure of the regulatory and catalytic Subunits of PKA

'P' = phosphorylation
'Myr' = myristoylation
bonds, however, were shown to be unnecessary for stable dimerization of the RI subunits (Leon et al 1997). RII dimerization domains, which lack disulfide bonding (Leon et al 1997), have unique "anchoring" motifs that enable the dimers, but not the individual subunits, to bind to A-kinase anchoring proteins (AKAPs) which localize the RII dimers to specific cellular locations and signaling complexes (reviewed in Colledge & Scott 1999). It is suggested that in both the RI and RII dimer interface hydrophobic interactions are important for stable dimerization and may involve residues Leu-13 and Phe-36 (Leon et al 1997).

The inhibitory region of the R-subunits is the site of interaction with the C-subunits. This domain contains a sequence that mimics the consensus phosphorylation sequence found in PKA substrates: Arg-Arg-Xaa-Xaa(P)-Xaa with (P) indicating the phosphorylation site (Kemp et al 1977; Poteet-Smith et al 1997). Substitution of either of the Arg residues with Ala resulted in increased Km values, highlighting the importance of multiple basic residues in determining substrate specificity (Kemp et al 1977). The RII subunit contains the sequence Arg-Arg-Val-Ser-Val that can be phosphorylated at the Ser by PKA. The RI subunit, by contrast, contains a pseudophosphorylation site, Arg-Arg-Gly-Ala/Gly-Ile, in which the Ser is substituted with a non-phosphorylatable Ala or Gly (Jahnsen et al 1986; Clegg et al 1988; reviewed in Francis & Corbin 1999). Ile98, adjacent to the pseudophosphorylation site in RI subunits has been shown to be important for the inhibition of C-subunits (Poteet-Smith et al 1997). The inhibitory site of the heat-stable inhibitor of PKA (PKI) contains a similar pseudophosphorylation site to RI subunits, allowing the inhibitor to also block the activity of C-subunits (Scott et al 1985).
The carboxyl terminus of the R-subunit contains two types of cAMP binding sites termed A and B, or fast-binding and slow-binding, respectively, based upon the rate of dissociation of bound cAMP (Doskeland 1978; Ogreid & Doskeland 1980). The cAMP binding sites are homologous to the bacterial cAMP-binding protein, catabolite gene-activating protein (CAP) (Bubis & Taylor 1987), for which the amino acid sequence and structure have been elucidated (McKay et al 1982). The domains consist of three α-helices and an eight-stranded β-barrel which form a binding pocket for cAMP (McKay et al 1982; Zorn et al 1995). cAMP binding domain A is directly involved in interactions with the C-subunits and is important for inhibiting the catalytic activity of the holoenzyme. Domain B, by contrast, is not involved in the inhibition of the C-subunit but rather regulates the binding of cAMP to domain A (Huang & Taylor 1998).

I.3.3.1 cAMP-Binding Characteristics

Binding of one molecule of cAMP to site B (slow-binding) of the R-subunit induces a conformational change in the subunit which in turn stimulates the binding of another molecule of cAMP to site A (fast-binding) of the same R-subunit (Doskeland & Ogreid 1981; Doskeland & Ogreid 1984). This positive cooperativity (Hill coefficients=1.4-1.6) (Bubis & Taylor 1987) is mainly due to intrachain interactions, but interchain cooperativity has been observed (Doskeland & Ogreid 1984). The interaction of the sites allows for low concentrations of cAMP to produce large increases in PKA activity in tissue (Ogreid & Doskeland 1980; Spaulding 1993; Tasken et al 1997). When two molecules of cAMP bind to each R-subunit, the affinity of the R-subunits for the C-subunits decreases by 10,000 - 100,000-fold, causing the dissociation of the holoenzyme into a dimer of R-subunits with
four bound molecules of cAMP, and two free catalytically active C-subunits (Taylor et al 1990; Francis & Corbin 1994; Tasken et al 1997). Cyclic nucleotide phosphodiesterases (PDEs) terminate the cAMP signal by hydrolyzing cAMP to 5'-adenosine monophosphate (5'AMP) (reviewed in Bushnik & Conti 1996), which dissociates from the R-subunit dimer, thereby allowing the R- and C-subunit holoenzyme to reassociate (Beebe 1994).

I.3.4 Catalytic Subunit

I.3.4.1 Structure

The X-ray crystallographic structure of the C-subunits of PKA was determined by Knighton et al (1991a, b) revealing a two-lobed structure of 350 amino acids with a deep cleft between the lobes (see Figure 2). The amino-terminal lobe, the smaller of the two, is dominated by β-strands, and is the site of Mg-ATP binding. The larger lobe, which includes the carboxyl-terminus is dominated by α-helices and is involved in substrate recognition and catalysis. Within the cleft of the subunit is the catalytic core that is conserved in all known protein kinases and consists of two converging β-sheets (Knighton et al 1991a, b; Tasken et al 1997). The carboxyl-terminus extends from the large lobe to the small lobe and is involved in nucleotide and substrate binding (Knighton et al 1991a, b; Smith et al 1999).

I.3.4.2 Co- and Post-Translational Modifications of Catalytic Subunits

The amino-terminal glycine residue of Cα and Cβ₁ is myristoylated and the group is an amide-linked n-tetradecanoic acid methyl ester that is surrounded by a hydrophobic pocket at the amino-terminus of the C-subunit (Carr et al 1982; Yonemoto et al 1993). The
role of this lipid modification is still under investigation, but there is evidence to suggest that myristoylation at the N-terminal may confer structural stability to the C-subunit (Yonemoto et al 1993). A second modification of the C-subunits has been discovered which involves the deamidation of Asn-2 to Asp-2 in the amino-terminus of the subunit. The deamidation is believed to account for differences in activity between the two main isoforms of C-subunits (α and β) and may be involved in the regulation of cellular localization (Jedrzejewski et al 1998).

The C-subunit, in contrast to the majority of other kinases, is fully phosphorylated in the holoenzyme state. Rather than the phosphorylation being the step that activates the enzyme, as in many other kinases (Taylor et al 1990), the dissociation of the holoenzyme and the release of free C-subunits produces the activation (Yonemoto et al 1997; Smith et al 1999). There are four sites for phosphorylation on the C-subunit: Ser-338, Thr-197, Ser-10, Ser-139. Analysis of mutant enzymes revealed that both Ser-338 and Thr-197 were stable phosphorylation sites. Ser-338 was shown to be important for the stability of the subunit. Thr-197 was shown to be necessary for proper catalytic activity and holoenzyme formation, and segment 191-199 has been named the ‘activation’ loop due to this involvement (Yonemoto et al 1997). While the phosphorylation sites can be autophosphorylated (Yonemoto et al 1997), heterologous phosphorylation on Thr-197 by an uncharacterized PKA kinase has been observed (Cauthron et al 1998). A recent study suggests that this PKA kinase may be phosphoinositide-dependent protein kinase (PDK1), suggesting a novel mechanism for the regulation of PKA (Cheng et al 1998).
I.3.5 Conformational Flexibility and Catalysis

The holoenzyme structure of PKA is an extended "dumbbell"-shaped structure. The dimerization of the R-subunits forms the bar, while the regions in contact with the C-subunits and the cAMP-binding domains extend outwards from the middle dimerization region. In this holoenzyme complex the C-subunits are in a "closed" formation and the R-subunits are highly extended (Zhao et al 1998). cAMP-binding to the R-subunits is postulated to be accompanied by a fold at the hinge region of the R-subunits, and the release of the C-subunits (Gibson & Taylor 1997; Huang & Taylor 1998; Zhao et al 1998). Free C-subunits, upon release from the R-subunit dimer, assume an "open" conformation in which the catalytic cleft is exposed for binding (Knighton et al 1991b; Zhao et al 1998).

The first step of catalysis involves the binding of Mg-ATP to the open cleft of the free C-subunit that switches the conformation of the C-subunit into a "closed" active site conformation for catalysis (Cheng et al 1998; Shaffer & Adams 1999; Smith et al 1999). Substrates with the consensus sequence (Arg-Arg-Xaa-Sermir(P)-Xaa) will bind to the active site cleft of the C-subunit in an extended coil conformation (Bramson et al 1987). The "closed" conformation and the positioning of the substrate and nucleotide allow the γ-phosphate of ATP to be in close proximity to the Ser/Thr residue, the site of phosphorylation. Asp-166 acts as the base-catalyst for the nucleophilic attack of the γ-phosphate by the Ser (Taylor et al 1990; Zheng et al 1993). The rate-limiting step, after the rapid phosphorylation of the substrate and release of the phosphopeptide, is the opening of the active site and the consequent release of Mg-ADP which would convert the C-subunit
back to the "open" conformation (Zheng et al. 1993; Shaffer & Adams 1999; Smith et al. 1999).

### I.3.6 Protein Phosphorylation Mediated by PKA

Protein phosphorylation by kinases like PKA is now recognized as an extremely important part of the regulation of biological processes. Changes in protein phosphorylation account for almost all of the actions of second messengers, and provide a means for extracellular signals to produce long-term changes in structure and function (Taylor et al. 1990; Girault 1993). Phosphorylation causes conformational changes and local "steric and charge effects" in proteins which activate or inactive a substrate protein, and in some cases alter the activity of the substrate protein (Nestler & Greengard 1983; Taylor et al. 1990; Girault 1993). Processes regulated by PKA-mediated phosphorylation include carbohydrate metabolism, lipid metabolism, protein synthesis, and a number of neuronal functions including neurotransmitter biosynthesis, axoplasmic transport, neurotransmitter release, postsynaptic potentials, ion channel conductance, neuronal outgrowth and migration, elaboration of dendritic and axonal processes, and development and differentiation (Nestler & Greengard 1983; reviewed in Girault 1993).

Many of the important changes brought about by PKA are carried out at the level of gene expression through the phosphorylation of transcription factors (Nestler & Greengard 1983; Montminy et al. 1990; Taylor et al. 1990; Daniel et al. 1998) including the cAMP response element binding protein (CREB), cAMP response element modulator (CREM), activating transcription factor-1 and NF-κB (reviewed in Daniel et al. 1998). The phosphorylation of transcription factors occurs following the translocation of free C-
subunits to the nucleus of the cell by passive diffusion through the nuclear pore complex (NPC) (Wolf et al 1999).

I.3.7 Characteristics of PKA Isoforms

There are distinct differences among the different PKA isoforms that are believed to contribute to the specificity of the cAMP second messenger system (Doskeland et al 1993; Spaulding 1993; Tasken et al 1997; Hansson et al 1999). The isoforms show unique tissue and subcellular distributions, functions and regulatory properties (reviewed in Francis & Corbin 1999; Hansson et al 1999).

I.3.7.1 Tissue/Subcellular Distribution

α-subunits (Cα, RIα, RIIα) are expressed in almost all tissue types, while the β-subunits (Cβ, RIβ, RIIβ) are found almost entirely in neural and endocrine tissues, although low-level expressions of the β-subunits can be seen in a wide range of human tissues (Uhler et al 1986a, b; Scott et al 1987; Clegg et al 1988; Doskeland et al 1993; Guthrie et al 1997; Brandon et al 1998). To date, Cγ-subunit expression has only been found in human testis (Beebe et al 1990; Tasken et al 1997). RII subunits, due to associations with AKAPs, are usually found localized to specific cellular structures and organelles including elements of the cytoskeleton, the Golgi complex and microtubule organizing centers (Deviller et al 1984; Carr et al 1992; Brandon et al 1998; reviewed in Colledge & Scott 1999). The RI subunits are normally found in the cytosolic fraction (Deviller et al 1984), however, increasing evidence suggests that RI subunits might also associate with various subcellular components through interaction with AKAPs (Huang et al 1997; Hansson et al 1999).
I.3.7.2 Interactions of Regulatory Subunits with Catalytic Subunits and cAMP

The subtypes of R- and C-subunits show distinctive interactions with each other and the R-subunits show unique responses and differential sensitivity to cAMP (Tasken et al 1997; Francis & Corbin 1999). RI subunits have a unique high-affinity Mg-ATP binding site in the amino-terminus. The occupation of the site enhances the binding of RI to C-subunits which stabilizes the holoenzyme and makes the kinase less sensitive to activation by cAMP (Doskeland et al 1993; Francis & Corbin 1994). The RII subunits of the PKA-II holoenzyme are uniquely autophosphorylated upon binding to C-subunits, the effect of which reduces the affinity of the two subunits for each other (reviewed in Taylor et al 1990; Francis & Corbin 1999). RII subunits in many situations have been shown to have a higher affinity for C-subunits than RI subunits (Otten & McKnight 1989; Hansson et al 1999). For example, the overexpression of RIIα in NIH3T3 cells caused increased levels of PKA-II and decreased levels of PKA-I. Overexpression of RIIα in the same cells, however, did not alter the levels of either holoenzyme (Otten & McKnight 1989).

Holoenzymes containing RIB are activated by 3-7-fold lower concentrations of cAMP than holoenzymes containing RIA. The increased sensitivity of the β isoform might be attributable to different amino acid sequences in the pseudosubstrate regions of the R-subunits. Similarly, holoenzymes made up of RIα-Cβ are activated by lower concentrations of cAMP than holoenzyme containing RIα-Cα (Cadd et al 1990). These findings suggest that the tissue-specific expression of the β-subunits, which are the predominant isoforms expressed in brain tissue, is the determinant of PKA sensitivity to cAMP stimulation (Cadd et al 1990; Brandon et al 1997; Tasken et al 1997).
The unique affinities of the subtypes of the R-subunits for the C-subunits and cAMP can result in differences in PKA activity (Spaulding 1993). For example, in rat brain it was shown that low levels of PKA activity were found in regions which contained high levels of RIIβ. Conversely, increased responsiveness to cAMP and high levels of PKA activity were found in brain regions containing low levels of RIIβ (Ventra et al 1996).

1.3.7.3 Subunit Functions

Differential functions have been attributed to the PKA-I and PKA-II holoenzymes (Doskeland et al 1993; Spaulding 1993; Hansson et al 1999). PKA-I is generally thought to be associated with the cytosolic fractions of cells (Deviller et al 1984) where it was assumed to interact freely with any circulating proteins (Hansson et al 1999). Recently, however, PKA-I was shown to be specifically involved in the inhibition of cAMP on cell replication in human T lymphocytes (Skalhegg & Tasken 1997). Furthermore, PKA-I but not PKA-II was shown to localize with the antigen receptor complex during anti-immunoglobulin-induced B cell activation (Levy et al 1996). These findings suggest that PKA-I is more strictly controlled than previously thought (Huang et al 1997; Hansson et al 1999). PKA-II is generally associated with AKAPs that are believed to anchor the RII subunits to specific subcellular structures, and thus specific substrates for phosphorylation (reviewed in Colledge & Scott 1999). Thus PKA-II holoenzymes are associated with many cellular processes including regulation of alpha-amino-3-hydroxy-5-methyl-4-isoazole-propionic acid (AMPA) /kainate Ca\(^{2+}\) channels (Rosenmund et al 1994), modulation of L-type Ca\(^{2+}\) channel activity (Fraser et al 1998), cAMP-mediated gene transcription (Cassano et al 1996) and activation of lipolysis in adipocytes (Beebe et al 1984).
The different subtypes of R- and C-subunits have also been shown to possess differential functions. RIIβ mutant mice showed severe deficits in the regulation of gene expression and in their ability to perform on a rotarod apparatus, a complicated motor task. This suggests that RIIβ may have an important role in both gene regulation and complex motor skills (Brandon et al 1998). RIIβ mutants have also exhibited a chronically lean phenotype attributed to increased amounts of PKA-I holoenzyme (Cummings et al 1996). Cβ1 mutant mice were deficient in long-term depression and depotentiation in hippocampal cells but showed normal viability and no pathological defects, suggesting the importance of the Cβ1 subunit in various learning processes (Qi et al 1996).

The subunits of PKA show unique patterns of expression during cellular differentiation (Tasken et al 1997; Hansson et al 1999). For example, during spermatogenesis the mRNAs for R1α, R1β and Cα were induced at premeiotic and meiotic stages. The mRNAs for R1I subunits were expressed at the haploid stages with R1Iα mRNA expression occurring even later than R1Iβ expression (Oyen et al 1990). These findings suggest that different PKA subunits might be responsible for discrete functions throughout cellular development (Oyen et al 1990; Tasken et al 1997).

I.3.7.4 Regulation of PKA Levels and Expression

PKA subunits show many different compensatory mechanisms in response to prolonged increases or decreases in the level of cAMP in order to regulate catalytic activity within cells (Spaulding 1993). The particular responses differ greatly depending upon the cell-type or tissue being studied (Spaulding 1993; Hansson et al 1999). In primary rat hepatocyte cultures, sustained elevations in cAMP caused a down-regulation of C-subunits
due to increased degradation. R-subunit levels were unchanged but there were increased levels of RIo and RIIo mRNA, as well as an increased R-/C-subunit ratio (Houge et al 1990). Ovarian granulosa cells exposed to estradiol and forskolin for 48 h showed increased levels of RIIβ mRNA (6-10-fold) followed by increased de novo synthesis of the isoform (5-7-fold) (Ratoosh et al 1987). In rat sertoli cells, cAMP increased RIIβ mRNA more than 50-fold in the first 24 h, while RIo, RIIo and Ca mRNAs were increased 3-, 4- and 2-fold, respectively, due to increased transcriptional activity (Oyen et al 1988; Landmark et al 1991; Tasken et al 1991). However, prolonged stimulation (36-48 h) with cAMP resulted in time-dependent decreases in RIIβ mRNA revealing a biphasic, possibly homeostatic response, to cAMP (Oyen et al 1988). Protein levels of RIo and RIIβ were increased (2-fold and 10-20-fold, respectively) (Landmark et al 1991; Tasken et al 1991). The induction of RIIβ, RIIo and Ca mRNA required protein synthesis whereas that of RIo did not, suggesting different mechanisms for the transcriptional regulation of RII and RI subunits (Tasken et al 1991). Interestingly, transfection of chloramphenicol acetyltransferase (CAT) plasmids containing the promoter regions of the RIIβ gene in Sertoli cells, but not in mouse neuroblastoma cells and Chinese hamster ovary cells, resulted in increased CAT activity in response to cAMP analogs or forskolin, despite the absence of a consensus CRE sequence in the promoter (Luo et al 1992). In a human B lymphoid cell line (Reh), by contrast, the promoter regions of the RIo gene were shown to contain a putative CRE sequence. Exposure of this cell line to cAMP showed a 5-6-fold increase of RIo1b mRNA, and a 40-50-fold increase of RIo1a mRNA, revealing differential regulatory mechanisms for the two RIo splice variants (Solberg et al 1997).
These studies highlight the complexity of the feedback mechanisms of cAMP levels on the different PKA subunits, and the influence of the tissue type on these mechanisms.

The creation of mice that are null mutant for specific PKA subunits has revealed some interesting regulatory properties of the subunits (Hansson et al. 1999). In adipose tissue of RIIβ mutant mice the levels of RIα increased dramatically due to increased incorporation into holoenzyme which stabilized the subunits. This compensatory response minimizes the degradation of C-subunits. Normally, as RIIβ subunits preferentially associate with C-subunits, free RIα subunits are rapidly degraded. In the mutant mice, however, it seems that RIα could successfully bind to the free C-subunits (Amieux et al. 1997). By contrast, in brain tissue of RIIβ mutant mice, in which RIIβ is normally the dominant isoform, there was much less compensation by RI and profound decreases in Ca and Cβ subunit levels due to increased degradation (Brandon et al. 1998). These contrasting findings highlight important tissue-specific differences in R-subunit compensatory responses (Spaulding 1993).

Together, the above-mentioned studies highlight the number of ways in which the R- to C-subunit ratios can change under different conditions. Evidence suggests that changes in the ratio, that is changes in the relative amounts of the R- and C-subunits, affect PKA activity, perhaps in a compensatory manner (Spaulding 1993). For example, in rabbit skeletal muscle it was shown that when the amount of R-subunit relative to C-subunits is increased the activation constant for cAMP activation of PKA is also increased (Schwechheimer & Hofmann 1977). In concordance with this notion, in Aplysia long-term sensitization, that is increased responsiveness of PKA to cAMP, is associated with a
decreased amount of R-subunits resulting in a decreased R- to C-subunit ratio (Greenberg et al 1987). Finally, in GH3 cells, 24 h of forskolin treatment resulted in increased degradation of C-subunits accompanied by decreased PKA activity (Richardson et al 1990). Thus, these studies highlight the association of changes in R- to C-subunit ratios and PKA activity.

I.4 Additional Modes of Regulation of cAMP-Mediated Signal Transduction

I.4.1 Positive/Negative Feedback on the cAMP Signal Transduction Pathway

While cAMP-mediated PKA activity through the signal transduction pathway results in compensatory responses at the level of the kinase itself, activity in the pathway also results in compensatory responses at other stages of signal transduction. At the level of the GPCRs, after activation, the receptors are uncoupled from G proteins and are desensitized through PKA- and G protein receptor kinase (GRK)-mediated mechanisms (Zastawny et al 1997; Lefkowitz 1998). PKA can phosphorylate specific Ser and Thr residues in the third cytoplasmic loop or carboxy-terminal tail of the GPCR leading to conformational changes that affect the interaction of the receptor with G proteins (Zastawny et al 1997). Furthermore, recent evidence suggests that the phosphorylation of the GPCRs by PKA can cause a switching of their coupling from Gs to Gi, indicating a novel role for the feedback activity of PKA on the receptors (Lefkowitz 1998; Luo et al 1999). GRKs phosphorylate Ser and Thr residues in the carboxyl-terminal tail of agonist-occupied GPCRs. The phosphorylated receptors in turn bind arrestins, a family of proteins that function to impede further receptor-G protein interaction (Zastawny et al 1997; Lefkowitz 1998).
Other potential sites for feedback regulation by PKA include G proteins, AC and CREB. In astroglial cells, it was shown that sustained levels of cAMP caused time-dependent increases in the levels of Gsα mRNA through a PKA-mediated mechanism (Karim et al 1994). AC type V contains several consensus sequences for phosphorylation by PKA, the effects of which inactivate the enzyme (Iwami et al 1995). Similarly, AC type VI is phosphorylated by PKA at a site that interferes with its interaction with the Gsα protein, thereby inhibiting AC (Chen et al 1997). The promoter region of CREB contains CREs and cAMP was shown to stimulate the expression of the transcription factor suggesting that CREB gene expression is positively autoregulated (Meyer et al 1993).

PKA activity can also influence the function of protein serine/threonine phosphatases (PSPs) and PDEs. These are important enzymes that are directly involved in regulating cAMP-mediated and other signal transduction pathways (Bushnik & Conti 1996; Price & Mumby 1999)

I.4.1.1 Protein Serine/Threonine Phosphatases

The phosphorylation of target proteins by PKA is a reversible process due to the activity of PSPs that dephosphorylate the phosphoproteins. PSPs can be divided into two major classes by their amino acid sequences, PP(protein phosphatase)P and PPM. The PPP class includes PP1, PP2A, PP2B/calcineurin (CaN), PP4, PP5, PP6, and PP7 (Price & Mumby 1999). PP2A dephosphorylates P-CREB and blocks CREB-mediated transcription from a PEPCK promoter (Wadzinski et al 1993). PSP activity is regulated by the targeting of the phosphatases to specific locations by various adaptor, anchoring and scaffold proteins (Pawson & Scott 1997; Schillace & Scott 1999). PSPs are also under the control
of second-messengers like cAMP, which inhibits PP1 through a PKA-mediated mechanism (Greengard et al 1998). Ca\(^{2+}\), another important second messenger, activates PP2B/CaN in cooperation with calmodulin (CaM) (Guerini 1997).

### I.4.1.2 Phosphodiesterases

PDEs play an important role in modulating the duration and amplitude of the cAMP signal through the hydrolysis of cAMP to 5'AMP. The superfamily of PDEs include seven families differentiated based upon kinetic characteristics, modes of regulation and substrate specificity. The cAMP-specific PDE family (cAMP-PDEs or PDE4s) are insensitive to cGMP and Ca\(^{2+}\)/CaM and include PDE4A, PDE4B, PDE4C and PDE4D, as well as additional splice variants. These PDE subtypes show differential regulation, and tissue and subcellular distributions (reviewed in Bushnik & Conti 1996). PDE4D3, a splice variant of a cAMP-PDE4D, is rapidly activated in rat thyroid cells by cAMP through PKA phosphorylation of a regulatory domain only found in this splice variant (Madelian & La Vigne 1996; Sette & Conti 1996). In human T-cells, forskolin-stimulated cAMP production increased PDE activity through increased expression of PDE4D1 and PDE4D2, and the decreased expression of the PDE4A isoform (Erdogan & Houslay 1997). These studies highlight the complex nature of the regulation of PDE4s, through both fast and slow mechanisms, which serve to regulate the cAMP signal.

### I.4.2 PKA Binding Proteins

One hypothesized mechanism through which signal-transduction systems regulate the activity of kinases is through AKAPs (Schillace & Scott 1999). PKA-II is known to be targeted to various intracellular structures and signaling complexes through interaction with
the anchoring proteins (reviewed in Colledge & Scott 1999). For example, AKAP 350 has been shown to target RII subunits to centrosomes in human lymphoblast cell lines (Keryer et al 1993). This targeting allows the enzymes to be located in close proximity to specific substrates, phosphatases and other kinases so that the appropriate control of signaling responses is attained. In this manner, cells can efficiently respond to extracellular signals with appropriate and specific intracellular responses (reviewed in Colledge & Scott 1999; Fraser & Scott 1999).

Characterization of the PKA-binding domain of AKAPs reveals an amphipathic α-helix formation believed to be common to all AKAPs which binds to hydrophobic pockets comprised of residues 1-23 within the dimerization domains of the RII dimers (Carr et al 1992; reviewed in Colledge & Scott 1999). A second region of the AKAPs consists of a “targeting sequence” that anchors the protein to specific intracellular structures (reviewed in Pawson & Scott 1999). The AKAPs have been shown to bind with similar affinity to both RII dimers and PKA-II holoenzyme (Carr et al 1992). Recently, a novel AKAP that interacts with both RI and RII dimers has been characterized. The discovery of this protein, D-AKAP1, suggests that both the dimerization domains and the functions of RI and RII might be more similar than originally thought (Huang et al 1997; Leon et al 1997).

The localization of AKAPs with PKA and various other structures and functional proteins suggests numerous roles for the diverse family of anchoring proteins (see Colledge & Scott 1999 for review). Overexpression of AKAP75 in A126 cell lines localized PKA-II to the cellular membrane and induced cAMP-mediated transcription, indicating a possible role for the binding proteins in the regulation of transcription (Cassano et al 1996). AKAP79 localizes PKA-II to postsynaptic densities in neurons and has also been shown to
bind and inhibit PP2B (CaN). This suggests a role for the binding proteins in regulating synaptic transmission (Coghlan et al 1995). AKAP79 has recently been shown to also bind and inhibit protein kinase C (PKC), the latter of which can be activated through Ca\(^{2+}\)-mediated signal transduction pathways (Faux et al 1999). The binding of anchoring proteins to different enzymes can either inhibit or activate the enzymes depending upon the specific AKAP (Colledge & Scott 1999). It has been suggested that AKAP-PKA-II complexes can only respond to high levels of cAMP and may mediate the nuclear response of PKA to cAMP. Conversely, soluble PKA-I and PKA-II isoforms may mediate the cytoplasmic responses to cAMP (Ventra et al 1996).

Until recently, C-subunits were not believed to be associated with any binding proteins. C-subunits have now been shown to associate with IκBs, proteins which bind to, and inhibit the activity of the transcription factor NF-κB. The interaction of the C-subunits with IκB-NF-κB complexes occurs at the ATP-binding domain of the C-subunits; the binding inhibits the catalytic activity of the subunits. Activators of NF-κB such as interleukin-1 (IL-1) degrade the IκBs and release the C-subunits from inhibition. The free C-subunits are thus activated by a cAMP-independent mechanism and can subsequently activate NF-κB through phosphorylation of the transcription factor (Zhong et al 1997). Recent evidence also indicates that C-subunits can bind to calveolin-1, a protein that is involved in the formation of calveolae that are vesicular invaginations of the plasma membrane. The binding of calveolin-1 was shown to inhibit the catalytic activity of the C-subunits (Razani et al 1999). These interactions of C-subunits with various cellular proteins indicate other potential mechanisms for regulating C-subunits independently of cAMP-mediated pathways (Zhong et al 1997; Razani et al 1999).
I.4.3 cAMP-Dependent Protein Kinase Heat-Stable Inhibitor

Free C-subunits can also bind to PKI, which, as mentioned previously, contains a pseudophosphorylation site similar to RI. This high-affinity binding (Ki<1 nM), allows the inhibitor to block the catalytic activity of Cα and Cβ (Scott et al 1985), but not of Cγ (Beebe 1994). The role of PKI-inhibition of C-subunits is still not fully understood and is complicated by the fact that intracellular concentrations of PKI are believed to be much lower than the C-subunit concentrations (Francis & Corbin 1999). Interestingly, PKI has a nuclear export signal (NES) which allows the inhibitor to bring C-subunits that have translocated to the nucleus back into the cytoplasm (Wiley et al 1999), although the signal for this event has yet to be discovered (Francis & Corbin 1999).

I.4.4 Rap1-Mediated Mitogen-Activated Protein Kinase Pathway

In addition to stimulation of PKA, cAMP activates another important downstream target. This target, Rap1, is a small GTP-binding protein that belongs to the ras family (de Rooij et al 1998; Kawasaki et al 1999; Roberson et al 1999). In its inactive state, Rap1 is bound to GTP (Kawasaki et al 1999). cAMP binds to sites on a guanine-nucleotide exchange-factor (GEF) which has been named Epac. Epac, in turn, will activate Rap1 by exchanging GTP for GDP (de Rooij et al 1998). In its active GTP-bound state, Rap1 can act through B-Raf to activate mitogen-activated protein kinase kinase (MEK) which in turn activates mitogen-activated protein kinase (MAPK). Amongst other actions, MAPK can phosphorylate CREB thereby influencing the transcription of genes regulated by CREs. The activation of Epac by cAMP represents a novel pathway through which cAMP can act, in addition to the cascade involving PKA (de Rooij et al 1998; Kawasaki et al 1999). Rap1
can also be directly phosphorylated by PKA that serves to activate the protein (de Rooij et al 1998).

I.5. Phosphoinositide- and Ca\textsuperscript{2+}-Mediated Pathways

Two other major signal transduction systems implicated in the pathophysiology of BD are the PI- and Ca\textsuperscript{2+}-mediated pathways. Similar to the cAMP-mediated signal transduction pathway, some GPCRs are coupled to the activation of phospholipase C (PLC), through the \( \alpha \) subunits of the Gq family, or the \( \beta \gamma \) subunits of the Gi and Go families (Exton 1997). Some subtypes of PLC can also be activated through association with tyrosine phosphorylated proteins, such as growth factor receptor tyrosine kinases (Exton 1997). The activation of PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) into two second messenger products, IP\textsubscript{3} and DAG (Dubovsky et al 1992; Berridge 1997). IP\textsubscript{3} mobilizes Ca\textsuperscript{2+} from intracellular stores into the cytosol (Berridge 1997) and DAG is an activator of PKC (Nishizuka 1989). PKC, like PKA, is a kinase involved in a multitude of processes such as gene expression and neuronal plasticity, through the phosphorylation of specific substrate proteins (Asaoka et al 1992).

In addition to the release from intracellular stores, intracellular Ca\textsuperscript{2+} can also be increased by the influx of Ca\textsuperscript{2+} through both voltage-, ligand-gated ion-specific and store-operated Ca\textsuperscript{2+} channels (Rasmussen 1989; Putney 1990; Berridge 1997). Cellular stimulation can increase intracellular Ca\textsuperscript{2+} from basal levels of 50-200 nM into the \( \mu \)M range. The rapid increase in intracellular Ca\textsuperscript{2+} shows both oscillatory patterns (Berridge 1997) and spatial organization that are believed to determine the specificity of the Ca\textsuperscript{2+} signal (Bugrim 1999).
The PI signal is sustained through the recycling of PI by dephosphorylation of components of the system allowing the regeneration of free inositol. The enzyme involved in this regeneration is inositol monophosphatase (IMPase) (Parthasarathy et al 1994). Various mechanisms contribute to restitution of intracellular Ca\(^{2+}\) levels to baseline levels, including a CaM-dependent membrane pump, Ca\(^{2+}\) -dependent ATPase and Na\(^{+}\) -Ca\(^{2+}\) exchange (reviewed in Dubovsky et al 1992).

I.5.1 Crosstalk Between cAMP-, Ca\(^{2+}\)-, and Phosphoinositide-Mediated Pathways

Functional interactions between the cAMP- and the Ca\(^{2+}\)- and PI-mediated signal transduction pathways allow for the integration and modulation of cellular signaling at many signal transduction levels (Hill & Kendall 1989; Port & Malbon 1993). Figure 3 illustrates schematically the levels and extent of crosstalk between these intracellular signal transduction systems. Many proteins involved in the Ca\(^{2+}\)- and PI-mediated pathways can be regulated through phosphorylation by PKA (Girault 1993). The \(\beta_2a\) subunit of the L type Ca\(^{2+}\) channels contains sites that are phosphorylated by PKA and serve to stimulate the Ca\(^{2+}\) current (Gerhardstein et al 1999). PKA has also been shown to phosphorylate various IP\(_3\) receptor subtypes thereby modulating Ca\(^{2+}\) flux, either increasing or decreasing it depending upon the cell type and IP\(_3\) receptor subtype involved (Bugrim 1999). There is also evidence to suggest that the phosphorylation of one or more GTP-binding proteins by PKA inhibits PLC activity and consequently decreases PI hydrolysis and Ca\(^{2+}\) release (Wen et al 1992). PKA also phosphorylates Ca\(^{2+}\)/CaM-dependent protein kinase (CaMK), a
Figure 3: Crosstalk between the CAMP-, phosphatidylinositol-, and Ca²⁺-mediated signal transduction systems.
kinase which is stimulated by the Ca$^{2+}$/CaM complex, resulting in the inhibition of the enzyme (Matsushita & Nairn 1999).

Further interactions between these transduction cascades occur through the modulation of protein phosphorylation by PP2B/CaN (Guerini 1997; Price & Mumby 1999). Stimulation of PKC activity modulates the mRNA levels of RIIβ and RIIα subunits in rat Sertoli cells (Tasken et al 1992). PKC plays an important role in regulating receptor sensitivity such as through phosphorylation of cardiac β-ARs (Limas & Limas 1985). PKC also phosphorylates Gs resulting in reduced receptor-mediated accumulation of cAMP (Hollingsworth & Daly 1987). At the level of gene transcription, CREB has been shown to be a substrate for CaMK which is activated by Ca$^{2+}$ (Sheng et al 1990; Dash et al 1991). CaMKIV phosphorylation activates CREB while CaMKII phosphorylation results in the inhibition of CREB (Sun et al 1994) indicating that CREB, a substrate for PKA (Daniel et al 1998), can mediate responses to both cAMP and Ca$^{2+}$. Interestingly, a depolarization-mediated Ca$^{2+}$ influx in PC12 cells was shown to activate the Rap1/B-Raf pathway through a PKA-mediated mechanism highlighting an important point for crosstalk between Ca$^{2+}$- and PKA-mediated signaling (Grewal et al 2000).

1.6 Summary of Signal Transduction Pathways

It is evident that the cAMP-mediated signal transduction pathway is a highly complex signaling cascade that works in concert with other signaling pathways to regulate cellular functions. PKA is an integral part of the cAMP-mediated cascade. The interactions of PKA with other functional proteins and the contributions of PKA to the regulation of cellular functions are profound (Beebe 1994; Smith et al 1999). As such,
abnormalities of PKA would be expected to affect normal cellular signaling possibly leading to pathophysiological states.

I. 7 Signal Transduction in Bipolar Affective Disorder

At the present time the etiology of BD is still poorly understood. The complex trait genetics and heterogeneity of the disorder have been major impediments in elucidating the pathophysiological basis of BD. Nonetheless, neurobiochemical evidence from clinical studies of BD patients, as well as findings from studies examining the possible effects of various therapies for BD has generated some important insight into the pathophysiological bases of BD, at least in so far as the potential involvement of second messenger mediated processes (Warsh & Li 1996; Li et al 2000; Warsh & Li 2000). The next section reviews the current body of experimental evidence for disturbances in signal transduction pathways in BD as well as the effects of mood-stabilizers on the signaling pathways.

I.7.1 Clinical Studies of Bipolar Affective Disorder and the cAMP-Mediated Signal Transduction Cascade

To explore possible abnormalities in receptor-activated signal transduction in BD, many studies have examined the levels and the basal and/or stimulated activity of GPCRs and G proteins in post-mortem brain and various peripheral cell models in both BD and normal subjects. A number of investigators have used mononuclear leukocytes (MNLs) as a cell model to probe these processes and found alterations such as reduced β-AR-stimulated AC activity in both unipolar and bipolar depressed patients (Extein et al 1979; Pandey et al 1979; Mann et al 1985, 1997). Later work using lymphoblast cell lines as a model found a reduced capacity to down-regulate β-ARs in response to prolonged agonist
stimulation in BD as compared with healthy controls (Kay et al 1993). Although changes in receptor sensitivity were accompanied by differences in receptor density in some studies involving a small number of patients, including some BD patients (Extein et al 1979; Jeanningros et al 1991) a much more rigorous study that included a larger number of depressed patients including BD patients found blunted isoproterenol-stimulated cAMP formation without changes in MNL β-AR densities (Mann et al 1985). Such findings are difficult to reconcile on the basis of a change restricted to the receptor, and are more readily interpreted in the context of disturbances in downstream signal transduction processes that also modulate receptor sensitivity. Although these disturbances were observed in MNLs from both major depressive disorder (MDD) and BD patients, they provided one of the first hints that postreceptor signal transduction disturbances may be operating in either one or both of these groups of mood disorders.

More compelling evidence favouring the abnormal signal transduction hypothesis of BD came with the ability to examine the levels of various protein components in signal transduction cascades more directly with immunoassay techniques, and their mRNA expression levels through Northern analysis and polymerase chain reaction (PCR) based assays (reviewed in Li et al 2000; Warsh & Li 2000). Thus, semiquantitative estimation of G protein levels in postmortem BD brain yielded the first direct observations implicating postreceptor disturbances in components of the transmembrane transduction apparatus (Warsh & Li 2000). Elevated levels of Gsα proteins were found in prefrontal, temporal and occipital cortex but not in hippocampus, thalamus or cerebellum of postmortem brain from patients with BD in comparison with matched non-psychiatric controls (Young et al 1993). Similar findings of increased levels of Gsα were shown in the frontal cortical
membrane of postmortem brains of BD patients in comparison with non-psychiatric controls (Friedman & Wang 1996). The increased levels of Gsα might be expected to cause hyperfunctionality of the cAMP signaling cascade, a view supported by several sets of observations. For example, increased Gsα expression was associated with increased accumulation of cAMP, phosphorylation of CREB and CRE-reporter gene transcriptional activity in human embryonic kidney cells (Yang et al 1997). Gsα protein function can be determined using forskolin, a hypotensive diterpene which binds to the cytoplasmic domains of AC and activates the enzyme (Tang & Hurley 1998) synergistically with Gsα (Alousi et al 1991). Interestingly, hyperfunctionality of the Gsα proteins, measured by increased cAMP formation in response to stimulation by forskolin, has been observed in autopsied temporal and occipital cortex of BD patients in comparison with controls (Young et al 1993). Furthermore, hyperfunctionality is also suggested by the increased agonist-induced binding of radiolabeled non-hydrolyzable GTP analogues to Gsα in postmortem brains of BD patients compared with control subjects (Friedman & Wang 1996). Together, the increased levels of Gsα and the hyperfunctionality of the proteins when compared with control subjects suggest a possibly overactive Gsα-mediated cAMP-signal transduction cascade in BD.

Abnormalities related to BD have also been postulated to occur downstream from G proteins and AC, at the level of the PKA enzyme. Rahman et al (1997) examined the binding of radiolabeled cAMP, which provides an index of the amount of the R-subunits of PKA, in the postmortem brain from BD patients and healthy controls. 3H-cAMP binding was significantly reduced in the cytosolic, but not the particulate fractions of frontal, temporal, occipital, and parietal cortex, cerebellum, and thalamus of postmortem brain
from BD patients compared with controls. This finding is consonant with the notion of a hyperfunctional cAMP signaling cascade as the decreased binding may indicate reduced R-subunit levels that might leave increased amounts of free C-subunits available for catalytic activity. Indeed, long-term increases in cAMP have been shown to cause compensatory changes in R-subunit protein and mRNA levels in a tissue-specific manner (Oyen et al 1988; Houge et al 1991; Landmark et al 1991; Tasken et al 1991; Spaulding 1993). It is possible, therefore, that upstream hyperfunctional cAMP-mediated signaling in BD might result in the altered regulation of R-subunits.

Fields et al (1999) recently examined the activity of PKA in postmortem brain from the same sample of BD patients and healthy controls used in the binding study (ie. Rahman et al 1997). It was shown that the PKA phosphorylation of an exogenous substrate in the cytosolic fraction of temporal cortex of BD patients was significantly higher than in healthy controls at maximally-stimulating concentrations of cAMP. Concentration-response relationships for cAMP were further characterized in those areas of postmortem brain that showed the significantly higher maximally-stimulated PKA activity compared with controls. Basal PKA activity in the cytosolic fraction of temporal cortex was found to be higher in BD postmortem brain compared with controls, and a decreased EC50 for cAMP was found in both the cytosolic and particulate fractions of BD temporal cortex compared with healthy controls. The lower EC50 in temporal cortex of BD patients suggests increased responsiveness to cAMP, possibly reflecting an altered stoichiometry of R- and C-subunits; that is reduced amounts of R-subunits leading to a relatively increased abundance of free and active C-subunits. This interpretation is consistent with the findings of decreased cAMP binding in the postmortem brains of BD patients (Rahman et al 1997).
Alternatively, the lower EC50 could indicate that the R-subunits in BD post-mortem brain have a reduced affinity for the C-subunits, or rather that the R-subunits have an increased affinity for cAMP. Taken together, the results of these studies indicate possible abnormalities in the function of PKA, and in the relative abundance of the subunits of PKA in BD, possibly secondary to a hyperfunctional cAMP-mediated signal transduction system.

The findings of hyperfunctional cAMP signaling in post-mortem BD brain are complemented by parallel clinical studies using peripheral blood cells. Schreiber et al (1991) have shown increased agonist-stimulated \[^3\text{H}\text{Gpp(NH)}_3\] binding in MNL membranes from manic but not euthymic BD patients. Elevated levels of \(G\alpha\) were also shown in the MNLs from depressed BD compared with MDD patients and healthy subjects (Young et al 1994), and in the MNLs from manic and/or lithium (Li)-treated euthymic BD patients compared with healthy subjects (Manji et al 1995a). Increased \(G\alpha\) levels were also demonstrated in the platelets of euthymic BP-I and BP-II patients compared with healthy subjects (Mitchell et al 1997) and in the platelets of manic and/or Li-treated euthymic BD patients compared with non-psychiatric controls (Manji et al 1995a). In Epstein-Barr virus (EBV)-immortalized B lymphoblasts similar elevations in \(G\alpha\) protein levels were found, although only in BP-II patients, and not BP-I patients (Emamghoreishi 1998).

Similar to the findings in the brain, dysregulation of PKA activity has also been reported in platelets. Perez et al (1995) reported significantly more radiolabeled phosphate incorporated into a low-molecular-weight protein band (22 kDa), believed to be Rap1, in
platelets from euthymic BD patients compared with control subjects. The results suggested that PKA activity in the platelets of euthymic BD subjects was elevated compared with controls and therefore, a possible state-independent marker for hyperfunctional cAMP signaling in BD. A more recent study by the same group (Perez et al 1999), however, examined the immunoreactivity of the subunits of PKA and of Rap1 in euthymic, manic, and depressed BD patients and healthy subjects. In this study, higher levels of C-subunits were found in platelet homogenates from both manic and depressed BD patients compared with euthymic patients and controls, suggesting that these changes reflected the state of illness of BD patients. Interestingly, these workers also found higher levels of Rap1 in the BD patient group compared with healthy subjects, but irrespective of state; this suggested that the Rap1 elevation was a potential trait-related marker of BD. This finding highlights the possibility that the increased phosphorylation of Rap1 in platelets of euthymic BD patients found in the previous study (ie. Perez et al 1995), might be caused by increased levels of the substrate protein itself, and not by the increased activity of PKA. As Rap1 can be activated by cAMP through a GEF independently of PKA (de Rooij et al 1998), abnormalities of Rap1 may also represent an important marker of a possibly hyperfunctional cAMP-mediated signal transduction cascade in BD.

An important issue that remains to be addressed is whether the noted changes in cAMP signaling in BD are trait- or state-dependent. The findings in postmortem brain and peripheral blood cells might be confounded by state-related factors such as circulating transmitters, hormones and medication. Indeed, some studies do suggest that the abnormalities found in BD patients might be related to the state of illness of the patients. For example, a study of G protein levels and activity in the MNLs of BD patients showed
that in manic BD patients $[^3$H]$Gpp(NH)p$ binding, and the immunoreactive levels of $G_{\alpha}$ and $G_{\delta}$ were significantly increased, while in depressed BD patients the measures were significantly decreased, in comparison to healthy subjects (Avissar et al 1997a). A second study replicated these findings in depressed BD patients and showed that the reductions in both functionality and quantity of the $G$ proteins in both bipolar and unipolar depressed patients significantly correlated with the severity of depressive symptoms (Avissar et al 1997b). These findings indicate the need to further study the putative disturbances in the cAMP-mediated signal transduction pathway in BD using models that will help to elucidate whether specific abnormalities are state- or trait- related. This issue is addressed by this study and will be discussed further in the section dealing with the research strategy (see I.8).

1.7.2 Clinical Studies of Bipolar Affective Disorder and the Phosphoinositide- and Ca$^{2+}$-Mediated Signal Transduction Cascade

Abnormalities in the PI- and Ca$^{2+}$-mediated pathways have also been observed in BD patients. GTP$\gamma$S-stimulated PI hydrolysis was reduced in the post-mortem occipital cortex of BD patients in comparison with matched control subjects (Jope et al 1996). The immunoreactive level of $G_{q\alpha/11}$, an important component of the PI pathway, was found to be significantly increased in the same brain region (Mathews et al 1997). In peripheral cell models, higher PIP$_2$ levels have been found in platelets of BD patients as compared with healthy controls (Brown et al 1993). Together the findings raise the possibility that compensatory adaptations may occur in the PI pathway in response to alterations in activity (Mathews et al 1997; Brown et al 1993). Other evidence implicating altered PI signaling
has been found in studies of lymphoblastoid cell lines from BD patients. Banks et al (1990) reported decreased inositol incorporation into PIs in B lymphoblastoid membranes from BD patients, and Shamir et al (1998), found lower IMPase activity, notably in Li responders, in cell lines from BD as compared with healthy subjects. At the level of PKC, in comparison to schizophrenic patients and healthy control subjects, platelet membrane to cytosolic PKC ratios were elevated in manic BD patients (Friedman et al 1993). Similar findings were found in post-mortem frontal cortex where membrane PKC activity measured by histone phosphorylation was higher in BD patients compared with healthy controls (Wang & Friedman 1996).

Increased basal intracellular Ca$^{2+}$ concentrations (intracellular $[\text{Ca}^{2+}]_B$) have been found in platelets and lymphocytes from manic and depressed BD patients in comparison to healthy controls (Dubovsky et al 1992; Dubovsky et al 1994). Furthermore, an ultrafiltrate of plasma obtained from BD patients did not raise intracellular $[\text{Ca}^{2+}]_B$ in platelets from healthy controls suggesting that the elevations in $\text{Ca}^{2+}$ in BD patients are not simply related to a circulating factor (Dubovsky et al 1994). Similar findings of increased intracellular $[\text{Ca}^{2+}]_B$ were observed in transformed B lymphoblasts of BP-I patients, and not of BP-II patients or of MDD patients when compared with healthy controls or non-mood disorder psychiatric patients (Emamghoreishi et al 1997). Together these findings support the notion of trait-related abnormalities in $\text{Ca}^{2+}$ homeostasis in BD patients. Furthermore, findings of lower isoproterenol-stimulated cAMP formation in transformed lymphoblasts from BP-I patients with high intracellular $[\text{Ca}^{2+}]_B$ compared with BP-I patients with normal intracellular $[\text{Ca}^{2+}]_B$ and with healthy subjects do suggest that there may be associations
between Ca$^{2+}$ homeostasis in BP-I patients and G protein-mediated signaling (Emamghoreishi et al in press) highlighting possible crosstalk disturbances in BD.

### I.7.3 Effects of Mood-Stabilizers on Signal Transduction Pathways

Studies of mood-stabilizing agents and their effects on the cAMP, PI and Ca$^{2+}$ signal transduction pathways in both normal subjects and BD patients, and in various animal models, provide substantial indirect support for the idea that abnormalities in signal transduction contribute to the pathophysiology of BD. Li has been the most common antimanic and mood-stabilizing agent used over the past 30-40 years for the treatment of BD. There is a distinct delay in onset of therapeutic effect of 1-2 weeks after initiation of mood-stabilizer treatment and substantial improvement usually occurs after 3-4 weeks. Patients who show classic manic episode features during the course of illness tend to respond better to Li than those with psychotic, dysphoric, mixed, or rapid-cycling features (Price & Heninger 1994; Manji et al 1996). More recently, investigations into other modalities of treatment have shown that anticonvulsant drugs including valproate (VPA) and carbamazepine (CBZ) and lamotrigine are also effective in both the acute and long-term treatment of BD (Bowden 1988). While Li, and other treatments are currently used to treat BD, the biochemical and molecular bases for their modes of action are not well understood. (Manji et al 1995b; Manji et al 1996; Jope 1999a, b).

The mood stabilizers in current use do not show consistent effects at the level of neurotransmitters. In fact, increasing evidence suggests that they exert their effects at the level of signal transduction (Hudson et al 1993). This is one possible explanation for the pattern of delayed response to treatment, "pervasive dampening or enhancing effects on
mood” and the short-term continuation of mood-stabilizing effects after the cessation of treatment (Risby et al 1991; Price & Heninger 1994; Manji et al 1996). In this regard, one of the current hypotheses for the therapeutic mechanism of action of mood stabilizing drugs suggests the importance of their simultaneous actions on several signal transduction pathways (Manji et al 1995b; Jope 1999a, b; Li et al 2000). A particularly interesting hypothesis recently advanced proposes a bimodal action for the mood stabilizers, notably Li, in which the drugs act to stabilize signal transduction processes within a normal range by increasing basal activities, and by decreasing stimulus-induced increases in activity (Jope 1999a, b).

There is substantial evidence that Li attenuates both post-receptor and receptor-mediated AC activity (Ebstein et al 1988; reviewed in Hudson et al 1993). Six weeks of Li treatment in rats reduced serotonin (5-HT)-stimulated increases in [35S]GTPγS binding to Gsα, Giα and Goα indicating an effect of Li on receptor-G protein coupling (Wang & Friedman 1999). Chronic Li administration has also been shown to decrease the mRNA levels of Gsα, Giα1, and Giα2 in rat cortex, while not affecting the immunoreactivity of the proteins (Li et al 1993).

Effects of Li on PKA levels and activity have produced conflicting results, possibly due to the different experimental paradigms employed. One study showed that 30 days of in vivo Li administration decreased cAMP-dependent phosphorylation of endogenous proteins in rat brain hippocampus (Casebolt & Jope 1991). Similarly, Li, in vitro, decreased cAMP-dependent phosphorylation of microtubule-associated protein 2 as well as the phosphorylation of heat-stable microtubule proteins by purified C-subunit, suggesting a
direct effect of Li on PKA (Mori et al 1996). These authors showed in a later study that 5 weeks of *in vivo* Li administration increased cAMP binding to PKA and immunoreactivity levels of R- and C- subunits of PKA in the soluble fractions of rat brain (Mori et al 1998). A study in human tissue which examined PKA activity in the platelets of euthymic BD patients and controls showed that 15 days of Li treatment increased both basal and cAMP-stimulated phosphorylation of endogenous proteins in the patients, but not in the controls (Zanardi et al 1997).

Together, these studies demonstrate the variety of effects of Li at therapeutically relevant concentrations on the cAMP signaling system. However, they also point out some of the difficulty in attempting to compare results obtained with different experimental paradigms and designs. More importantly, however, the studies highlight that Li, one of the most effective mood-stabilizer treatments for BD patients, clearly has a number of effects on the cAMP-mediated second messenger systems, and notably PKA. Whether the observed effects are important for the therapeutic efficacy of Li remains to be established unequivocally (Jope et al 1999a), but they add further compelling support for the involvement of the cAMP-mediated signal transduction pathway in BD.

### I.8 Research Strategy

I have presented in this overview the body of observations pointing to abnormalities in the cAMP-mediated signal transduction pathway, as well as in the PI- and Ca\(^{2+}\)-mediated pathways in the pathophysiology of BD. More specifically, several lines of evidence suggest that the cAMP-mediated signal transduction cascade may be in a hyperfunctional state in BD, as noted. Recent evidence from human postmortem brain and cell model
investigations (Perez et al 1995; Rahman et al 1997; Fields et al 1999) as well as that from studies of Li action (see section I.7.2), suggests that these disturbances may in turn impact at the level of the principal target of the cAMP-mediated signal transduction pathway, PKA. Specifically, the findings suggest that there may be increased catalytic activity of PKA in brain and some peripheral cell types from BD patients which would be manifested by increased phosphorylation of substrate proteins in response to cAMP stimulation. As mentioned previously, it is not possible to address trait- versus state-related issues using platelets as a cellular model or post-mortem brain. Accordingly, a central objective of this thesis was to test the hypothesis that PKA activity is increased in BD using an appropriate cellular model, described in the next section, that would exclude state-related factors. This would allow for the elucidation of possible trait-related abnormalities of PKA in BD.

I.8.1 Cellular Model for the Assay of PKA Activity

The measurement of the activity of an enzyme such as PKA is not yet feasible within the living human brain. Furthermore, it is difficult to determine the levels of cAMP in postmortem brain as cAMP is rapidly degraded in this environment (Jones & Stavinoha 1979). Therefore, it was necessary to use alternative methods to test the hypotheses of this study. The development of analogous cellular models is one particular approach that allows the examination of enzymatic activity in cells which in certain circumstances may reflect alterations that occur in neurons. B-lymphocytes are peripheral blood cells that may be useful for this purpose, that is, as surrogates to study some aspects of cellular functions that may also be expressed in neurons. B-lymphocytes express β-AR which upon activation couple to Gs proteins with subsequent stimulation of AC and ultimately, PKA (Warsh et al 1988; Werstiuk et al 1990). The use of lymphocytes as an analogous cellular
model to study PKA activity is supported by observations indicating that similar changes in Gsα levels (increased in BD) and functionality occur in leukocyte preparations (Schreiber et al 1991; Young et al 1994) and postmortem brain (Young et al 1993) of BD patients. Reduced β-AR responsivity (Extein et al 1979; Pandey et al 1979; Mann et al 1985, 1997) and abnormalities of intracellular Ca^{2+} (Dubovsky et al 1994) in MNLs of BD patients in comparison to healthy controls further support the notion that putative signal transduction abnormalities in BD are manifested in some cells/tissues outside the CNS. Together the findings support the utility of B lymphocytes as a model for exploring the molecular basis for some of the signaling changes that have been observed in recent studies of the pathophysiology of BD.

As discussed, an issue that merits specific attention is that of the degree to which state-dependent factors contribute to the pathophysiological changes in cAMP signal transduction reported in BD. The use of the transformed B lymphoblast cell line (BLCL) model, in comparison to non-transformed peripheral cells, provides a potentially important research strategy to test whether certain putative cellular abnormalities are trait-related. This is possible arguably as the model permits the cell lines to be grown and expanded ex vivo segregated from in vivo state-related factors such as circulating levels of plasma catecholamines and/or stress hormones, circadian variation, effects of long-term drug treatment, exercise and diet, which might cause or contribute to observed changes in signal transduction pathways in vivo. From a pragmatic perspective the B lymphoblast model also addresses the difficulties inherent in recruiting sufficient numbers of drug-free BD patients to study signal transduction processes in affected individuals in the absence of the potential confounding effects of drug treatment.
An increasing body of evidence supports the validity of this research strategy to investigate signal transduction processes in BD. Recent studies have shown abnormalities in the levels of Gsα (Emamghoreishi 1998), Ca\(^{2+}\) homeostasis (Emamghoreishi et al. 1997), agonist-induced downregulation of β-AR density (Kay et al. 1993), Na\(^{+}\), K\(^{+}\)-ATPase activities (Cherry & Swann 1994), incorporation of inositol into PIs (Banks et al. 1990) and IMPase activity (Shamir et al. 1998) in BLCLs from BD patients in comparison to control subjects. Indeed, some of the findings in BLCLs concur with findings in peripheral cells and post-mortem brain (see section I.7) suggesting that BLCLs are a valid model for the study of signal transduction disturbances in BD. The utility of the model is also supported by studies of hypertensive subjects in which the BLCL model has proven efficacious in discovering trait-related disturbances. BLCLs from hypertensive patients have shown increased intracellular \([Ca^{2+}]_i\) in response to stimulation by platelet-activating factor (Gruska et al. 1997) and enhanced agonist-stimulated G protein activation (Siffert et al. 1995) in comparison to normotensive controls, correlating with the findings in peripheral blood cells (Gruska et al. 1997). Together, these studies highlight the potential of the BLCL model as an adjunctive tool for the elucidation of potential trait-related disturbances in cAMP-mediated signal transduction in BD. Accordingly, Epstein-Barr virus (EBV)-immortalized BLCLs will be used to examine the hypothesis that PKA activity is altered specifically in BD.

### I.8.2 Ca\(^{2+}\) Endophenotype

The measurement of BLCL intracellular \([Ca^{2+}]_i\) levels revealed that BP-I patients (or a subset of these patients) showed levels that were significantly higher compared to
those found in the BLCLs from psychiatric and non-psychiatric comparison groups, as discussed earlier (Emamghoreishi et al 1997). More recent analysis of a larger data set indicates that those BP-I patients with high intracellular $[\text{Ca}^{2+}]_B$ levels showed an absence of comorbid psychiatric diagnoses. This suggests that the high BLCL intracellular $[\text{Ca}^{2+}]_B$ may reflect a trait within the BP-I diagnostic group with some common pathophysiological disturbance. This may define a putative endophenotype within the BP-I patients. While the validity of the $\text{Ca}^{2+}$ endophenotype remains to be ascertained unequivocally, our group has examined BP-I patients with high intracellular $[\text{Ca}^{2+}]_B$ as a distinctive group, along with BP-I patients in general. That is, the BP-I patients are divided based upon their $\text{Ca}^{2+}$ levels into two groups. The first group consists of those BP-I patients with high intracellular $[\text{Ca}^{2+}]_B$ levels, defined as $\geq$ two standard deviations (SDs) above the mean of a healthy comparison group. The second group consists of those BP-I patients with "normal intracellular $[\text{Ca}^{2+}]_B$ levels", that is levels that are within two SDs of the values of the healthy control group. It is hypothesized that this putative endophenotype based on intracellular $[\text{Ca}^{2+}]_B$ levels will allow the differentiation of possibly clinically relevant subtypes of BD. Indeed, the findings of differences in isoproterenol-stimulated cAMP production between BLCLs from high and normal intracellular $[\text{Ca}^{2+}]_B$ BP-I patients further supports the division of the BP-I patients (Emamghoreishi et al in press). Furthermore, the interactions of the $\text{Ca}^{2+}$- and cAMP-mediated signal transduction pathways (see 1.5.1.1) suggest that alterations in $\text{Ca}^{2+}$ homeostasis might be associated with alterations in PKA activity.
I.8.3 Objectives and Hypotheses

The principal objectives of this study were to determine if cAMP-stimulated PKA activity in BLCLs of BD patients differs from that in psychiatric and healthy comparison subjects and, if so, whether the differences are associated with observed alterations in intracellular Ca\(^{2+}\) homeostasis in this disorder.

The specific hypotheses tested in this thesis are:

1) Maximally-stimulated PKA activity will be higher in BLCLs from BD patients compared with psychiatric comparison groups and healthy subjects.

2) Basal PKA activity will be increased in BLCLs from BD patients compared with psychiatric comparison groups and healthy subjects.

3) The EC50 for cAMP activation of PKA will be lower in BLCLs from BD patients compared with psychiatric comparison groups and healthy subjects.

4) The alterations in PKA activity will be related to the Ca\(^{2+}\) endophenotype of the BD patients. That is, those BD patients in the high intracellular [Ca\(^{2+}\)]\(_{B}\) group will show more pronounced alterations in BLCL PKA activity compared to those BD patients in the normal intracellular [Ca\(^{2+}\)]\(_{B}\) group.

5) Basal and maximally-stimulated PKA activity will be lower in MDD patients compared with psychiatric comparison groups and healthy subjects.
CHAPTER II
MATERIALS AND METHODS
II.1 Materials

II.1.1 Chemicals/Materials

The following chemicals/materials were purchased from Sigma chemical company (St. Louis, MO): RPMI-1640 containing 2 mM L-glutamine, bovine serum albumin (BSA), Trizma Base (Tris [hydroxymethyl]aminomethane), 2-mercaptoethanol (2-Hydroxyethylmercaptan; β-mercaptoethanol), Magnesium Chloride, Leupeptin, cAMP (adenosine 3'5'-cyclic monophosphate/Tris salt, IBMX (3-isobutyl-1-methylxanthine), Ficoll-Hypaque gradient and protein assay kit (Lowry reagent). cAMP-dependent protein kinase assay kit, P81 Phosphocellulose Squares, Kemptide Phosphate Acceptor Peptide and cAMP-dependent Protein Kinase Inhibitor Peptide (PKI(5-22)amide (TYADFIASGRTRNAINH₂) were purchased from Upstate Biotechnology (Lake Placid, NY). Fetal calf serum (FCS), AlM-V, penicillin (10,000 units/ml)/streptomycin (10 ug/ml), Phosphocellulose Disk Sheets and membrane filtered trypan blue (0.4% in 0.85% saline) were purchased from GibcoBRL (Burlington, ON). Acetone and Phosphoric Acid were obtained from Caledon (Georgetown, ON). The following chemicals/materials were purchased from Calbiochem (La Jolla, CA): AEBSF ([4-(2-Aminoethyl)-benzenesulfonylfluoride, Hydrochloride]) and KT5823 (a cGMPdk inhibitor). EDTA (Ethylenedinitrile)-tetracetic Disodium Salt) was purchased from Matheson, Coleman and Bell (Norwood, OH). A liquid scintillation counting solution comprised of an emulsifier and fluors (Ready-safe Liquid Scintillation Cocktail), was purchased from Beckman (Fullerton, CA). ATP[γ-33P] was purchased from New England Nuclear (Boston, MA). Protein assay dye (Bradford reagent) was obtained from Bio-Rad (Hercules, CA). T75
tissue culture flasks with vented caps were purchased from Sarstedt (St. Leonard, Quebec). Cyclosporine was a gift from Novartis (Dorval, Quebec). Epstein-Barr virus (EBV)-expressing B95-8 monkey kidney cells were obtained from the Department of Genetics, Hospital for Sick Children research foundation (Toronto, ON).

II.1.2 Buffers and Solutions

Ficoll-Hypaque gradient was prepared as a 60% solution (vol/vol) in RPMI-1640 for isolation of MNLs from whole blood. Media used for growing lymphoblasts contained (vol/vol) RPMI-1640 78%; FCS 20%; antibiotic (penicillin 104 units/ml; streptomycin 104 μg/ml) 1%; and sodium pyruvate 1%.

A TME buffer (20 mM Trizma Base, pH 7.4, 2 mM EDTA, 25 mM 2-mercaptoethanol) containing 0.5 mM AEBSF and 10 μg/ml leupeptin, was used for the PKA activity assays.

II.2 Subjects

Patients were recruited from the Bipolar and Mood Disorder Clinics of the Centre for Addiction and Mental Health, Clarke Division, and the Mood Disorders Program of the University of Toronto. Public advertising through newspapers, newsletters and various public forums was employed as a recruitment strategy for healthy subjects. Patients were enrolled in the study sequentially as they presented, irrespective of state and severity of illness. Inclusion criteria for the healthy subjects were: age 18-65; no current or past history of psychiatric disorder based on a structured interview with the Structured Clinical Interview for DSM-IV Axis I Disorders-Non-Patient (SCID-NP) version (First et al 1995);
no family history of psychiatric disorder, diabetes or hypertension in first-degree relatives; no abnormalities on physical examination, or in hematology and clinical biochemistry; no history of drug usage within the past 3 weeks; and provision of informed consent. Patient inclusion criteria were: age 18-65; a DSM-IV research diagnosis of BD (BP-I or BP-II) or MDD; physically healthy; no lifetime or family (first-degree relatives) history of diabetes or hypertension; and no recent (>3 months) drug or alcohol abuse. Patients provided informed consent and were clinically assessed by their psychiatrist. Psychiatric diagnoses were confirmed according to the criteria delineated above using the SCID version IV (First et al 1995) administered by a research psychiatrist or trained psychiatric nurse, and complemented by review of available medical records. Present state and symptom severity in affective disorder patients were assessed using the Hamilton Depression (HAM-D) (17-item) (Hamilton 1960; Williams 1988) or Beck Depression Inventory (BDI) (21-item version) rating scales for depression, or Clinician Administered Rating Scale for Mania (CARS-M) (Altman et al 1994) for hypomania/mania. All diagnostic information was reviewed by a research psychiatrist to confirm the clinical diagnosis.

Patients and comparison subjects were physically healthy based on history and functional inquiry. The venipuncture was performed on the day of diagnostic assessment.

Cell lines from patients and healthy subjects were categorized into groups of either BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\) and healthy control (N=16 per group), or BP-II, MDD and healthy control (N = 10 per group) for the PKA activity assays. Patient samples were also matched on sex and age of the respective control subjects and duration of time that the cells were in storage, for subsequent biochemical assays.
The study was approved by the Human Subjects Review Committee of the University of Toronto and, after a complete description of the study, all subjects provided written informed consent.

II.3 Isolation of Mixed Leukocytes and B Lymphocyte Transformation

Blood (0-15 ml) was collected by antecubital venipuncture into vacutainers containing acid citrate dextrose as an anticoagulant, held at room temperature (RT), and processed within 24 h of venipuncture. MNLs were isolated as previously described (Emamghoreishi et al 1997). Blood was centrifuged at 100 g for 20 min at RT. The platelet-rich plasma was removed and the lower layer of sedimented red and white blood cells was mixed with RPMI-1640 (1:3 vol/vol). Eight ml of diluted cells were layered over a Ficoll-Hypaque gradient and centrifuged at 400 g for 30 min at RT (Boyum 1968). The interface layer containing mostly MNLs was transferred, pooled, diluted with an equal volume of RPMI-1640, and recentrifuged as above for 10 min. The pellet was washed with RPMI-1640 and resuspended in the same medium. After counting with an electronic cell counter (Coulter), an aliquot of MNLs (4 x 10^6 cells) was resuspended in 1 ml RPMI-1640 containing 2 mM L-glutamine, 1 mM pyruvate, 20% FCS, 100 μg/ml streptomycin and 100 units/ml penicillin. 0.5 ml of filtered supernatant from an EBV-expressing B95-8 monkey kidney cell line culture and 1.3 μg/ml cyclosporine A were added, and the cells incubated at 37°C in a 95% air/5% CO₂ humidified incubator for two weeks. Once the cell lines were established, the cells were transferred and grown in suspension in the same medium in T-25 tissue culture flasks. The cells were ‘passaged’ every 2-3 days by adding an equivalent volume of fresh medium. After 12-15 passages, the cells were sedimented (400 g, 10 min),
resuspended in 10 ml RPMI and washed (400 g, 10 min). Cells were then resuspended into aliquots of 30–100 x 10^6 cells and washed again (400 g, 2 min). The washings were decanted and the remaining pellets were flash-frozen in liquid nitrogen and held at -70°C until future assay. Cell viability was greater than 90% at the time of harvest, as determined by trypan blue exclusion.

II.4 Optimization of the PKA Activity Assay

An extensive literature search did not reveal any previously published work on PKA activity in BLCLs. Therefore, initial experiments were conducted to optimize the assay conditions of basal and cAMP-stimulated PKA activity in these cell lines prior to the determination of PKA activity in BLCLs from patient and comparison groups.

II.4.1 Method for Determining PKA-Specific Activity

Two basic approaches have been used to determine PKA-specific activity in tissues and cell lines. One method uses inhibitors of kinases other than PKA to block the "non-specific" phosphorylation of a peptide substrate added to the reaction mixture. The other employs a specific inhibitor of PKA to determine the activity that is attributable to PKA. In the former method, based on the technique by Roskoski (1983), with minor changes (cAMP-dependent protein kinase assay kit, Upstate Biotechnology), a cocktail of inhibitors is used which contains a PKC inhibitor peptide (2 μM) and the CaMk inhibitor compound R24571. The PKA-specific activity is calculated by subtracting a substrate (Kemptide)-free/inhibitor-cocktail condition from a substrate/inhibitor-cocktail condition. This method assumes that protein phosphorylation by kinases other than PKA are blocked by the inhibitor-cocktail. Given the incomplete knowledge of all potential kinases present in the
BLCLs it is still not possible to prepare an inhibitor-cocktail that contains all of the necessary selective inhibitors to ensure specific and complete inhibition of those kinases that would phosphorylate the peptide substrate used (i.e. Kemptide). Furthermore, it is now well established that Kemptide, with an affinity of 5 μM for PKA (Kemp et al 1977), also serves as a substrate for cGMP-dependent protein kinase (cGMPdk) (Km = 231 μM) (Glass et al 1986). cGMPdk is also stimulated by cAMP (Ka = 0.08 μM and 0.3 μM for PKA and cGMPdk, respectively) (Torphy et al 1982). These facts suggested that the phosphotransferase activity measured by this method might overestimate that activity which is actually PKA-specific and might in fact be attributable to cGMPdk.

To establish the conditions providing the greatest specificity of the assay for BLCL PKA activity, a series of assays were carried out to compare the substrate/inhibitor-cocktail method and an alternative method which uses the PKA-specific inhibitor, PKI(5-22)amide (5 μM), to define the phosphotransferase activity that is specific to PKA. PKI(5-22)amide, a synthetic peptide of the naturally occurring inhibitor of PKA, PKI, is a potent and highly specific inhibitor for PKA (Ki = 3 nM) (Glass et al 1989; Kemp et al 1991; Walsh & Glass 1991). The inhibitor has a pseudosubstrate moiety that competes with substrate for binding to free C-subunits (Glass et al 1989). The use of PKI to measure PKA-specific activity involves determining the degree of substrate phosphorylation in the absence and presence of PKI and subsequently calculating the difference in activities between the two conditions. cAMP concentrations of 1 μM were used in the assays to measure PKA-specific activity.
To analyze the potential contribution of cGMPdk to the estimated phosphotransferase activity, a cGMPdk-specific inhibitor KT5823 (Kase et al 1987) (1-2 μM) was included in some assays.

II.4.2 Selection of Fresh vs. Frozen Sample Preparation

The BLCL model afforded the possibility of using fresh living cells for the estimation of enzyme activity thereby circumventing possible freezing and storage effects in the determination of enzyme activity. However, it is extremely difficult, if not impossible logistically, to utilize fresh cells without freezing the cells and at the same time control for variables such as age of the subjects, sex, passage number of the cells and interassay variability. The preferred experimental design is one that controls for these variables by matching subjects and assaying the matched subject samples in parallel. The use of a frozen cell preparation allows the comparison of samples from subjects matched on sex, age and passage number of the cells within the same assay, as cell lines matched on these variables can be harvested after similar passage numbers and frozen until a later date for assay. Therefore, frozen BLCLs were used for this study and the effects of freezing and duration of storage on PKA activity were assessed statistically.

II.4.3 PKA Activity Assay

BLCL PKA activity was determined using a modification of the procedure of Roskoski et al (1983). Frozen BLCLs were thawed slowly on ice and then homogenized by ultrasonication (sonicator duty cycle setting 30%, micro tip limit 3, 80s; Sonics and Materials, Danbury, CT, USA) in 10 ml/10⁶ cells of ice cold TME buffer (20 mM Tris-HCl pH 7.4; 2 mM EDTA; 25 mM 2-mercaptoethanol) containing 0.5 mM AEBSF and 10
μg/ml leupeptin). The homogenates were centrifuged (48,000 g, 30 min, 4 °C), and the resulting supernatants were used as a crude cytosolic fraction. Protein concentrations were determined by the method of Bradford (Bradford 1976) using Coomassie Brilliant Blue G-250 as the protein bound dye reagent. Bovine serum albumin was used as the standard. 10 μl of the cytosolic fraction containing approximately 5 μg of protein was mixed in duplicate with 10 μl each of: 100 μM Kemptide, varying concentrations of cAMP (2-500 nM) where indicated, TME buffer with or without 5 μM of PKI(5-22)amide (final concentrations are indicated). Samples were pre-incubated (30°C, 5 min) to allow the reagents to reach the temperature required for the reaction (Murray 1995). 10 μl of Mg\(^{2+}\) (18.75mM)/ATP (125 μM) cocktail containing 1 μCi ATP [γ-\(^{33}\)P] was added and the samples were incubated for an additional 10 min at 30°C. Then, 25 μl of each sample reaction mixture were pipetted onto P81 phosphocellulose paper and the papers were air dried (5 or 30 min depending on sample ordering). Free \(^{33}\)P orthophosphate and ATP[γ-\(^{33}\)P] were removed by washing the papers 5 times for 5 min, each, with 100 ml of 0.75% phosphoric acid by shaking, and the papers were dried by rinsing twice for 2 min, each, with 100 ml of acetone. Bound \(^{33}\)P-Kemptide was then quantified by scintillation spectrometry. Sample radioactivity was corrected for a counting efficiency of 98% and expressed as disintegrations per minute (dpm). Specific PKA activity was determined by subtracting the activity which was not inhibitable by PKI(5-22)amide from the total activity. All samples from each cell line were adjusted for protein amount (varied with cell line) and reaction time (10 min), so that activity was expressed as pmol/min/μg protein. Appropriate blanks in which the 10 μl of cytosolic fraction was replaced with 10 μl of TME buffer were included to determine non-specific, bound radioactivity.
II.4.4 Additional Preliminary Experiments for Optimization of PKA Activity

II.4.4.1 Cytosolic and Particulate Fractions – Centrifugal Force

In the absence of published data on the particular subtypes of PKA expressed in BLCLs, and their subcellular localization, preliminary experiments were undertaken to establish the relative PKA activities in the cytosolic and particulate fractions to guide decisions on the fractions to be assayed in the clinical research design. Assays were conducted in which BLCL homogenates were fractionated using centrifugal forces of 48,000g or 100,000 g, which have been widely used in the literature to prepare cytosolic and particulate fractions for biochemical study (Cassano et al 1996; Fields et al 1999). cAMP (1 μM)-stimulated PKA activity was measured in each subcellular fraction as described above to determine the optimal conditions for subsequent assays of samples from comparison subjects.

II.4.4.2 Concentration-Response Relationship of PKA Activity for cAMP

The response of PKA activity to cAMP stimulation was established in a series of preliminary experiments in which cAMP concentrations were varied from 2 nM to 10 μM to determine the maximally stimulating concentration.

II.4.4.3 Inhibition of Phosphodiesterase Activity

3-Isobutyl-1-Methylxanthine (IBMX) is a non-specific competitive inhibitor of cAMP PDEs (Beavo et al 1970; Montague & Cook 1971) used by some investigators in assays of PKA activity to inhibit the degradation of cAMP by PDEs in tissues with relatively high PDE activity (Tomes et al 1993). To assess the necessity for inclusion of a
PDE inhibitor in the assay of PKA activity in BLCLs, comparisons of both basal and maximally-stimulated ([cAMP]=500 nM) PKA activity were performed in the presence and absence of 500 μM IBMX, a concentration widely established to produce a high degree (90%) of PDE inhibition (Beavo et al 1970).

II.4.4.4 Concentration-Response Relationship of PKA Activity for Kemptide

Kemptide, a widely used substrate for PKA exhibits a Km of 16 μM for purified PKA from bovine skeletal muscle (Kemp et al 1977). It is highly specific for PKA and poorly phosphorylated by other kinases (e.g. cGMPdk, Km = 231 μM) (Glass et al 1986). The relationship between PKA activity and Kemptide concentration was evaluated in a series of experiments in which varying concentrations of Kemptide (2 μM– 1 mM) were used with 500 nM cAMP to confirm the choice of an optimal concentration of Kemptide for subsequent assays of BLCLs from patients and controls.

II.4.4.5 Linearity of PKA Activity with Respect to Protein

Basal and maximally-stimulated ([cAMP]=500 nM) PKA activity were assayed as described with varying amounts of BLCL cytosolic fraction protein (2–20 μg) to determine the range over which PKA activity was directly proportional to protein amount for this preparation. The protein amount at the mid-point of the linear range of activity was then used in subsequent assays of samples from comparison subjects.
II.4.4.6 Linearity of PKA Activity with Respect to Time

Linearity of both basal and maximally-stimulated ([cAMP]=500 nM) PKA activity for a range of incubation times was evaluated to ensure a sampling time point for the assays that was within the linear range of PKA activity with respect to time.

II.5 Characterization of PKA Activity in BLCLs

Based on the results of these preliminary, confirmatory studies the following experimental assay protocol and assay conditions as described above (II.4.3) were used for the subsequent comparison of PKA activity in BLCLs from patients and healthy subjects. In each assay, the PKA activity of three BLCLs from three subjects (a BP-I High Ca$^{2+}$ patient and BP-I Normal Ca$^{2+}$ patient and a healthy subject, or a MDD and BP-II patient and a healthy subject) matched by sex and age were included. Concentration-response relationships of cAMP-stimulated PKA activity for each of the three cell lines were determined in duplicate within the single assay. The basal PKA activity, maximally-stimulated ([cAMP]=500 nM) PKA activity, as well as the EC50 for cAMP activation of PKA were characterized for each of the three cell lines.

II. 6 Statistical Analyses

Data are expressed as mean ± SD throughout the thesis.

Analysis of the concentration-response data for cAMP was performed using nonlinear, reiterative curve fitting based upon the minimization of the sum of the square of the vertical distances of the data points from the calculated line and determined using the method of Levenberg-Marquardt (Marquardt 1963) with the GraphPad Prism 3.0 software.
cAMP concentrations were log-transformed for the curve fitting procedure. A sigmoidal dose-response equation was chosen as this function provides the most physiologically relevant mathematical description of the relationship and empirically provides the best fit model for the generated data. Due to the positive cooperativity that exists for the two cAMP-binding sites of the R-subunit of PKA the variable slope option was chosen for the sigmoidal dose-response equation.

Differences in the dependent measures (basal PKA activity, maximally-stimulated PKA activity or EC50 for cAMP activation of PKA) for the different methodological aspects of the assay that were addressed were assessed by the non-parametric Wilcoxon matched-pairs signed-ranks test due to small sample sizes. Differences in dependent measures (basal PKA activity, maximal PKA activity and EC50 for cAMP activation of PKA) between comparison groups were assessed by univariate ANOVAs with diagnosis and sex as factors. Differences in dependent measures for patients stratified by state of illness (illness state-depressed, manic/hypomaniac or euthymic), lifetime history of a comorbid psychiatric disorder (presence or absence) and the type of comorbid diagnosis (anxiety disorder or substance abuse) were assessed by univariate ANOVAs with diagnosis, sex and the particular stratification variable as factors. Appropriate designs were constructed for each of the ANOVAs so that the interactions of specific factors were included in the design only if there were adequate numbers of subjects to support the analyses. For patients stratified by the age of onset factor (early (≤19 years) or adult (≥20 years)) differences in maximally stimulated PKA activity were assessed using the Student’s t-test. Tukey post-hoc tests were employed when necessary to test for differences in cell means within factors where significant main effects were obtained. Potential correlations
of dependent PKA activity measures with age, age of onset, sex, intracellular $[\text{Ca}^{2+}]_B$ and duration of freezer storage were assessed using the Pearson product-moment test and a significant correlation was further investigated using an ANCOVA to assess the covariance of the factor. Two-tailed probability values of $p < 0.05$ were set to distinguish statistically significant differences. Statistical analyses were performed using the SPSS (release 10.0) statistical software package.
CHAPTER III

RESULTS
III.1 Subjects

Table 3 summarizes the demographic characteristics of the subjects who participated in the study. The mean age of the subject groups studied varied from 33.9 ± 9.51 years for the BP-I Normal Ca²⁺ patients to 38.0 ± 11.8 years for the BP-II group. There were no statistically significant differences in age among comparison groups (F=0.839, df=4,66 p=0.505). A statistically significant main effect of age of onset was found among the comparison groups (F=5.615, df=3,36, p<0.05). Tukey post-hoc comparison of cell means revealed that the age of onset of the MDD patients (30.4 ± 15.2) was significantly greater than that of the BP-I High Ca²⁺ (15.5 ± 5.08; p<0.01), BP-I Normal Ca²⁺ (19.75 ± 4.79; p<0.05) and BP-II patients (19 ± 6.76; p<0.05). Seventy percent of the MDD patients, 50% of the BP-II patients and 20% of the BP-I Normal Ca²⁺ patients were medication free. Seventy percent of the BP-I High Ca²⁺ patients, 60% of the BP-I Normal Ca²⁺ and BP-II patients, and 20% of the MDD patients were euthymic at the time of study. A chi-square test revealed that the four patient groups were not significantly different in the proportions who were euthymic or ill (χ²= 5.24, df=3, p=0.20). Fifty percent of the BP-I High Ca²⁺ patients, 60% of the BP-I Normal Ca²⁺ patients and BP-II patients, and 30% of MDD patients had a lifetime history of a comorbid anxiety disorder (Panic Disorder, Generalized Anxiety Disorder, Posttraumatic Stress Disorder, Obsessive-Compulsive Disorder, Anorexia Nervosa) or substance abuse (Alcohol, Cannabis, Sedative). A chi-square test revealed that the four patient groups did not different significantly in the percentages of those patients with a comorbid diagnosis (χ²=2.93, df=3, p=1.0).
Table 3- Characteristics of healthy subjects, and BP-I High Ca^{2+}, BP-I Normal Ca^{2+}, BP-II and MDD patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy N=19</th>
<th>BP-I High Ca^{2+} N=16</th>
<th>BP-I Normal Ca^{2+} N=16</th>
<th>BP-II N=10</th>
<th>MDD N=10</th>
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<td>Sex</td>
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</tr>
<tr>
<td></td>
<td>Female</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>36.7 ± 10.7</td>
<td>34.0 ± 10.7</td>
<td>33.9 ± 9.51</td>
<td>38.0 ± 11.8</td>
<td>37.9 ± 11.4</td>
</tr>
<tr>
<td>Age of onset (mean ± SD)</td>
<td>N/A ^a</td>
<td>15.5 ± 5.08</td>
<td>19.8 ± 4.79</td>
<td>19.0 ± 6.76</td>
<td>30.4 ± 15.2 ^b</td>
</tr>
<tr>
<td>Mood state</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Depressed</td>
<td>N/A</td>
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<td>6</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Manic/hypomanic</td>
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<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Euthymic</td>
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<td>11</td>
<td>9</td>
<td>6</td>
<td>3</td>
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<td>Comorbidity</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Anxiety Disorders</td>
<td>N/A</td>
<td>3</td>
<td>2</td>
<td>3</td>
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<tr>
<td>Substance Abuse</td>
<td></td>
<td>5</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Medication Free</td>
<td>N/A</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

^a Not applicable

^b Age of onset for MDD patients is significantly greater (p<0.05) than that for the BP-I High Ca^{2+}, BP-I Normal Ca^{2+} and BP-II patients
III.2  Optimization of the PKA Activity Assay

III.2.1 Method for Determining PKA-Specific Activity

A comparison was made between the assays of phosphotransferase activity in the BLCL cytosolic fraction using the substrate/inhibitor-cocktail method and the PKA inhibitor method. As shown in Figure 4, the assay procedure based on the substrate/inhibitor-cocktail method gave slightly higher estimates of cAMP (1 μM)-stimulated PKA activity compared with the assay using the PKA inhibitor method, although this was not statistically significant (0.36 ± 0.44 vs 0.30 ± 0.44 pmol/min/μg protein, Wilcoxon test, w=-1.604, p=0.109, n=3). Figure 5 shows that increasing the concentration of PKI(5-22)amide to 10 μM did not result in higher PKA-specific activity indicating that complete inhibition of PKA by PKI(5-22)amide occurred at a concentration of 5 μM. Figure 6 shows the portion of total activity which was resistant to inhibition by PKI(5-22)amide and the portion of activity which was inhibited by PKI(5-22)amide and thus PKA-specific, for various concentrations of cAMP. In the basal condition, approximately 40% of total activity was inhibited by the inhibitor while in the cAMP-stimulated condition approximately 80% of total activity was attenuated. Of note, the absolute PKI(5-22)amide-resistant activity increased (30-50%) from basal to stimulating concentrations of cAMP. This observation suggested that there may be a PKI(5-22)amide-resistant kinase activated by cAMP. To explore whether cGMPdk is responsible for the cAMP-activated PKI(5-22)amide-resistant phosphotransferase activity, the assay was performed in the presence of a cGMPdk inhibitor, KT5823. Figure 7 shows that the cAMP (1 μM)-stimulated activity measured in the presence of the cGMPdk-specific inhibitor (1-2
Figure 4: Comparison of cAMP (1 μM)-stimulated PKA-specific activity using the Inhibitor Method and the Substrate/Inhibitor-Cocktail Method in the cytosolic fraction of BLCLs as described in the methods. Data are expressed as mean ± SD for 3 samples assayed in duplicate.
PKA-specific activity with [PKI(5-22)amide]=5 μM

PKA-specific activity with [PKI(5-22)amide]=10 μM

Figure 5: Comparison of PKI(5-22)amide concentration (5 and 10 μM) cAMP (1 μM)-stimulated PKA activity in the cytosolic fractions of 2 BLCLs assayed in duplicate with [Kemptide]=100 μM.
Figure 6: Representative sample of PKA activity for different [cAMP] in a BLCL assayed in duplicate as described in the methods. For each [cAMP], the first bar represents the total activity while the second bar shows the fraction of the total activity in the presence of PKI(5-22)amide (PKI(5-22)amide-resistant, striped bar), and that which is inhibited by PKI(5-22)amide (PKA-specific activity, solid bar).
Figure 7: Comparison of the effect of 1 μM and 2 μM [KT5823] (cGMPdk-specific inhibitor) on PKI(5-22)amide-resistant activity in the cytosolic fraction of a representative BLCL from a healthy subject assayed in duplicate.
μM) was not different from the activity measured in the absence of the inhibitor. The effects of higher concentrations of the inhibitor were not explored due to the known inhibition of PKA by the cGMPdk-specific inhibitor (Ki >10 μM) at high concentrations.

III.2.2 Cytosolic and Particulate Fractions – Centrifugal Force

Initial experiments revealed very low PKA activity levels in the post 48,000 g particulate fractions. As shown in the concentration-response relationship for cAMP in Figure 8, cAMP (1 μM)-stimulated PKA activity in the particulate fraction of a representative cell line was approximately 30% of that measured in the cytosolic fraction. Moreover, the activity in the particulate fraction did not exhibit a concentration-response relationship for cAMP. Results from the experiments in which the PKA activity of the fractions obtained using a centrifugal force of 48,000 g was compared with that obtained using a centrifugal force of 100,000 g are shown in Figure 9. Cytosolic PKA activity was 38% higher in the samples fractionated at 48,000 g (2.09 ± 0.84 pmol/min/μg protein) than that in samples fractionated using the 100,000 g (1.30 ± 0.48 pmol/min/μg protein) condition, although the difference was not statistically significant (Wilcoxon test, w=-1.60, p=0.109, n=3). The particulate PKA activity obtained using the 48,000 g (0.22 ± 0.13 pmol/min/μg protein) and the 100,000 g (0.24 ± 0.10 pmol/min/μg protein) conditions was very similar and did not reach significance (Wilcoxon test, w=-1.07, p=0.285, n=3). These results indicated that the loss of activity in the cytosolic fraction found with increasing the centrifugal force was not correspondingly accounted for in the particulate fraction.
Figure 8: Representative comparison of the log concentration-response relationships of PKA activity for cAMP stimulation in the cytosolic and particulate fractions of a BLCL assayed in duplicate as described in the methods. A centrifugal force of 48,000 g was used to separate the BLCL into the cytosolic and particulate fractions.
Cytosolic Fraction Activity (pmol/min/mg protein)

Particulate Fraction Activity (pmol/min/mg protein)

Figure 9: Comparison of centriuged forces for the determination of cAMP 1

Assayed in duplicate.

BLCs as described in the methods. Data are expressed as mean ± SD for 3 samples. (A) Simultaneous PKA activity in both the cytosolic (□) and particulate (▪) fractions of 1

Centriugal Force (g)
III.2.3 Concentration-Response Relationship of PKA Activity for cAMP

Figure 10 shows a typical concentration-response relationship for stimulation of cytosolic PKA activity by cAMP (0–500 nM). The concentration response curve of PKA activity was best fit by a typical sigmoidal log concentration-response relationship. For the subject sample shown in Figure 10, the calculated basal PKA activity and maximally-stimulated PKA activity, and EC50 for cAMP activation of PKA were 0.20 pmol/min/µg protein, 3.8 pmol/min/µg protein and 26.1 nM respectively. At 500 nM of cAMP a maximum level of PKA activity was obtained which was 19-fold that of the basal PKA activity. The Hill Slope for the sample subject was 1.87 and is within 1 SD of the mean Hill Slope of the healthy subjects (1.71 ± 0.21, N=16). These Hill slope values concur with the known cooperative nature of the cAMP binding.

III.2.4 Inhibition of Phosphodiesterase Activity

Figure 11 shows the cytosolic PKA activity measured in the presence and absence of the non-specific PDE inhibitor IBMX. In the basal condition, PKA activity was not significantly different in the presence (0.73 ± 0.14 pmol/min/µg protein) or absence (0.73 ± 0.12 pmol/min/µg protein) of IBMX (Wilcoxon test, w=0.000, p=1.0, n=3). While the results were not statistically significant (Wilcoxon test, w=-1.604, p=0.11, n=3), there was a trend towards higher PKA activity in the absence of IBMX (2.15 ± 0.96 pmol/min/µg protein) than in the presence of IBMX (1.89 ± 0.89 pmol/min/µg protein) in the maximally-stimulated ([cAMP]=500 nM) condition.
Figure 10: A representative log concentration-response relationship of cytosolic PKA activity for cAMP stimulation in a BLCL from a healthy subject assayed in duplicate as described in the methods. The relationship is fit to a sigmoidal dose-response equation with Hill Slope = 1.87 and is within 1 SD of the mean Hill slope of the healthy subjects (1.71 ± 0.21). Basal activity, maximally-stimulated PKA activity, and EC50 for cAMP activation of PKA were 0.20 pmol/min/μg protein, 3.8 pmol/min/μg protein and 26.10 respectively.
methods. Data are expressed as mean ± SD for 3 samples assayed in duplicate.

Figure 11: Effect of IBMX on the basal (A) and maximally-stimulated (B) PKA activity in BLCLs determined as described in the text.
### III.2.5 Concentration-Response Relationship of PKA Activity for Kemptide

Figure 12 shows a representative relationship of BLCL cytosolic maximally-stimulated ([cAMP]=500 nM) PKA activity and the concentration of substrate, Kemptide (2 μM – 1 mM). In this cell line, the relationship was best fit with a one-site binding hyperbolic equation ($r^2=0.84$) with $K_m=9.3 \text{ μM}$ and the $V_{max}=1.61 \text{ pmol/min/μg protein}$. A series of experiments revealed that the average $K_m$ for Kemptide in BLCLs is $11.1 \pm 1.7 \text{ μM (n=3)}$.

### III.2.6 Linearity of PKA Activity with Respect to Protein

Typical concentration-response relationships of cytosolic fraction PKA activity for protein in both the basal and maximally-stimulated ([cAMP]=500 nM) conditions are shown in Figure 13. Both basal and maximally-stimulated PKA activity showed increases with respect to the amount of protein. The fits of the protein-response relationships of both basal and maximally-stimulated PKA activity with first-order (linear) and second-order polynomial equations were compared. In the basal condition the relationship was best fit with a first-order (linear) polynomial equation ($r^2=0.94$, $F=0.80$, $df=1,4$, $p=0.42$), while in the maximally-stimulated condition the relationship was best fit with a second-order polynomial equation ($r^2=0.98$, $F=13.41$, $df=1,7$, $p=0.008$). The better least square fit obtained in the maximally-stimulated condition with a second-order polynomial equation was essentially attributable to “saturation” effects at protein amounts > 8 μg.
**Figure 12:** A representative concentration-response relationship of maximally-stimulated ([cAMP]=500 nM) PKA activity for [Kemptide]. The relationship is fit to a one-site binding (hyperbolic) equation with $K_m=9.3$ and $V_{max}=1.61$. 
Figure 13: Representative relationship of basal (A) and maximally-stimulated (B) PKA activity with respect to protein amount in the cytosolic fraction of a BLCL assayed in duplicate measured as described in the methods. The fits of the relationships of both basal and maximally-stimulated ([cAMP]=500 nM) PKA activity and protein amount with first-order (linear) and second-order polynomial equations were compared. The basal condition was best fit with a first-order polynomial equation ($r^2=0.94$, $F=0.80$, $df=1.4$, $p=0.42$). The maximally-stimulated condition was best fit with a second-order polynomial equation ($r^2=0.98$, $F=13.41$, $df=1.7$, $p=0.008$).
III.2.7 Linearity of PKA Activity with Respect to Time

Exploration of the relationship of PKA activity with respect to time revealed linear increases in both basal and maximally-stimulated ([cAMP]=500 nM) PKA activity (basal \( r^2=0.98 \); maximally-stimulated: \( r^2=0.96 \); see Figure 14) with respect to time over the 20 min time interval studied. Based on the findings, a 10 min incubation time was chosen for subsequent experiments.

III.3 Characterization of PKA Activity in BLCLs

Due to constraints on BLCL availability and subject matching criteria, BLCLs of seven of the controls used in the BP-I High Ca\(^{2+}\) and BP-I Normal Ca\(^{2+}\) comparisons were used in the MDD and BP-II comparisons. As such, the analysis of the BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\) and healthy subjects were initially conducted separately from the analysis of the MDD, BP-II and healthy subjects.

III.3.1 Comparison of BLCL PKA Activity in BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\) and Healthy Subjects

Table 4 shows the basal PKA activity, maximally-stimulated ([cAMP]=500 nM) PKA activity, and EC50 values for cAMP activation of PKA in BLCLs from the BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\) and healthy subjects matched on age and sex. As the initial assays revealed no significant differences in the EC50 for cAMP activation of PKA amongst the comparison groups (n=12 in each group), and determination of full dose-response relationships was laborious and costly, only the basal and maximally-stimulated PKA activities were determined for the cell lines in the four remaining sets of subjects.
Figure 14: Representative relationship of basal (A) and maximally-stimulated ([cAMP]=500 nM) (B) PKA activity with respect to time in a BLCL assayed in duplicate as described in the methods. The relationships are fit with first-order (linear) polynomial equations (basal condition: $r^2=0.98$, maximally-stimulated condition: $r^2=0.96$).
Table 4- BLCL cytosolic fraction PKA activity parameters in healthy subjects and BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), or BD (BP-I High Ca\(^{2+}\) and BP-I Normal Ca\(^{2+}\)) patients

<table>
<thead>
<tr>
<th>Diagnostic Group</th>
<th>Basal Activity (pmol/min/μg)</th>
<th>Maximally-Stimulated Activity (pmol/min/μg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Mean SD</td>
<td>N Mean SD</td>
<td>N Mean SD</td>
</tr>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>7 0.19 0.11</td>
<td>8 3.03 1.03</td>
<td>6 29.3 3.76</td>
</tr>
<tr>
<td>Male</td>
<td>8 0.15 0.08</td>
<td>8 3.51 1.28</td>
<td>6 31.7 3.99</td>
</tr>
<tr>
<td>Total</td>
<td>15 0.16 0.09</td>
<td>16 3.28 1.14</td>
<td>12 30.5 3.89</td>
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<tr>
<td>BP-I High Ca(^{2+})</td>
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</tr>
<tr>
<td>Female</td>
<td>8 0.19 0.11</td>
<td>8 3.14 0.65</td>
<td>6 27.9 6.10</td>
</tr>
<tr>
<td>Male</td>
<td>8 0.18 0.08</td>
<td>8 2.84 0.51</td>
<td>6 28.2 5.73</td>
</tr>
<tr>
<td>Total</td>
<td>16 0.18 0.10</td>
<td>16 2.99 0.59</td>
<td>12 28.1 5.64</td>
</tr>
<tr>
<td>BP-I Norm Ca(^{2+})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8 0.16 0.06</td>
<td>8 3.30 0.80</td>
<td>6 29.8 2.99</td>
</tr>
<tr>
<td>Male</td>
<td>7 0.19 0.13</td>
<td>8 2.96 0.75</td>
<td>5 33.1 3.40</td>
</tr>
<tr>
<td>Total</td>
<td>15 0.18 0.09</td>
<td>16 3.13 0.76</td>
<td>11 31.3 3.45</td>
</tr>
<tr>
<td>BD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>16 0.18 0.09</td>
<td>16 3.21 0.71</td>
<td>12 28.9 4.69</td>
</tr>
<tr>
<td>Male</td>
<td>15 0.18 0.10</td>
<td>16 2.90 0.63</td>
<td>11 30.4 5.24</td>
</tr>
<tr>
<td>Total</td>
<td>31 0.18 0.09</td>
<td>32 3.06 0.68</td>
<td>23 29.6 4.91</td>
</tr>
</tbody>
</table>
Furthermore, for two subjects (a BP-I Normal Ca\(^{2+}\) patient and one healthy subject) the calculated basal activities were negative and thus their results for the variable of basal PKA activity were excluded from further analyses. Given the different numbers of samples with measurable values for the three dependent variables (basal PKA activity, maximally-stimulated PKA activity and EC50 for cAMP activation of PKA), separate univariate ANOVAs were conducted for the variables in order to accommodate the requirements of the statistical analysis.

ANOVA revealed no significant main effects of diagnosis (F=0.041, df=2,40, p=0.960), and sex (F=0.115, df=1,40, p=0.737), nor interaction of diagnosis and sex (F=0.437, df=2,40, p=0.649) on basal PKA activity. There were also no significant main effects of diagnosis (F=0.419, df=2, 42, p=0.661), and sex (F=0.038, df=1, 42, p=0.846), and no interaction between these factors (F=1.164, df=2,42, p=0.322) on maximally-stimulated PKA activity, although, maximally-stimulated PKA activity was slightly lower (9\%) in BP-I High Ca\(^{2+}\) patients compared with healthy subjects. Furthermore, there were no significant effects of diagnosis (F=1.738, df=2,29, p=0.194), sex (F=1.609, df=1,29, p=0.215), nor interaction of diagnosis and sex (F=0.324, df=2,29, p=0.726) on the EC50 for cAMP activation of PKA.

To rule out the possibility that stratification of the patients based on intracellular [Ca\(^{2+}\)]\(_{\text{b}}\) masked possible relevant differences between the patients and healthy subjects, the BP-I High Ca\(^{2+}\) and BP-I Normal Ca\(^{2+}\) patients were collapsed into a single BD patient group and compared with the healthy subjects. Separate univariate ANOVAs for the basal
PKA activity, maximally-stimulated PKA activity and the EC50 for cAMP activation of PKA did not reveal any significant effects, however (see Table 4).

Results from BP-I High Ca^{2+} and BP-I Normal Ca^{2+} patients stratified based on state (illness state-depressed, manic/hypomanic or euthymic) were also analyzed to rule out a possible effect of state of illness on PKA activity (see Table 5). A univariate ANOVA with maximally-stimulated PKA activity as the dependent variable, and state, diagnosis and sex as factors showed a trend for a significant main effect of sex ($F=3.729, \text{df}=1,25, p=0.065$), while the main effects of diagnosis and state were not significant ($F=0.752, \text{df}=1,25, p=0.394$ and $F=2.279, \text{df}=1,25, p=0.144$, respectively). Although just a trend, an examination of the means showed that when state is included as a factor the female patients showed somewhat higher maximally-stimulated PKA activities than the male patients ($3.21 \pm 0.71$ and $2.90 \pm 0.63$ pmol/min/µg protein, respectively). There were no significant differences in basal activity or EC50 values in BD patient groups stratified by state.

As noted in the overview, about 40% of BD patients have a lifetime history of some other comorbid psychiatric disorder, particularly anxiety disorders and substance abuse. For this reason analyses were performed for the BP-I High Ca^{2+} and BP-I Normal Ca^{2+} groups stratified based on whether or not the patients had a lifetime history of a comorbid diagnosis of either an anxiety disorder or substance abuse (see Table 6). Univariate ANOVAs of the basal PKA activities, maximally-stimulated PKA activities, and EC50s for cAMP activation of PKA did not show any statistically significant main effects or interactions for the patients stratified by the presence or absence of a comorbid diagnosis. The results for patients with comorbid diagnoses were further analyzed after stratifying by
Table 5 - Effect of state of illness on BLCL cytosolic fraction PKA activity from BP-I patients with High or Normal Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Sex and Diagnosis</th>
<th>State</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP-I High Ca(^{2+})</td>
<td>Ill State</td>
<td>5</td>
<td>0.11</td>
<td>0.06</td>
<td>5</td>
<td>3.13</td>
<td>0.54</td>
<td>3</td>
<td>30.2</td>
<td>5.96</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>11</td>
<td>0.20</td>
<td>0.10</td>
<td>11</td>
<td>2.93</td>
<td>0.63</td>
<td>9</td>
<td>27.3</td>
<td>5.70</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>0.18</td>
<td>0.10</td>
<td>16</td>
<td>2.99</td>
<td>0.59</td>
<td>12</td>
<td>28.1</td>
<td>5.64</td>
</tr>
<tr>
<td>BP-I Norm Ca(^{2+})</td>
<td>Ill State</td>
<td>7</td>
<td>0.19</td>
<td>0.13</td>
<td>7</td>
<td>3.35</td>
<td>1.04</td>
<td>6</td>
<td>31.6</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>8</td>
<td>0.15</td>
<td>0.05</td>
<td>9</td>
<td>2.95</td>
<td>0.48</td>
<td>5</td>
<td>30.9</td>
<td>4.26</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>15</td>
<td>0.18</td>
<td>0.09</td>
<td>16</td>
<td>3.13</td>
<td>0.76</td>
<td>11</td>
<td>31.3</td>
<td>3.45</td>
</tr>
<tr>
<td>Total Female</td>
<td>Ill State</td>
<td>5</td>
<td>0.15</td>
<td>0.08</td>
<td>5</td>
<td>3.63</td>
<td>0.88</td>
<td>4</td>
<td>31.4</td>
<td>5.13</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>11</td>
<td>0.19</td>
<td>0.10</td>
<td>11</td>
<td>3.04</td>
<td>0.58</td>
<td>8</td>
<td>27.6</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>0.18</td>
<td>0.09</td>
<td>16</td>
<td>3.21</td>
<td>0.71</td>
<td>12</td>
<td>28.9</td>
<td>4.69</td>
</tr>
<tr>
<td>Total Male</td>
<td>Ill State</td>
<td>7</td>
<td>0.18</td>
<td>0.13</td>
<td>7</td>
<td>3.00</td>
<td>0.76</td>
<td>5</td>
<td>31.0</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>8</td>
<td>0.19</td>
<td>0.08</td>
<td>9</td>
<td>2.83</td>
<td>0.53</td>
<td>6</td>
<td>29.9</td>
<td>6.80</td>
</tr>
<tr>
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<td>Total</td>
<td>15</td>
<td>0.18</td>
<td>0.10</td>
<td>16</td>
<td>2.90</td>
<td>0.63</td>
<td>11</td>
<td>30.4</td>
<td>5.24</td>
</tr>
<tr>
<td>Total</td>
<td>Ill State</td>
<td>12</td>
<td>0.16</td>
<td>0.11</td>
<td>12</td>
<td>3.26</td>
<td>0.84</td>
<td>9</td>
<td>31.2</td>
<td>3.88</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>19</td>
<td>0.19</td>
<td>0.09</td>
<td>20</td>
<td>2.94</td>
<td>0.55</td>
<td>14</td>
<td>28.6</td>
<td>5.36</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>31</td>
<td>0.18</td>
<td>0.09</td>
<td>32</td>
<td>3.06</td>
<td>0.68</td>
<td>23</td>
<td>29.6</td>
<td>4.91</td>
</tr>
</tbody>
</table>

\(^{a}\) trend for higher maximally-stimulated PKA activity in female BP-I patients stratified by state compared to male BP-I patients stratified by state (F=3.729, df=1,25, p=0.065)
Table 6 – BLCL cytosolic fraction PKA activity in BP-I patients with and without a lifetime history of psychiatric comorbidity

<table>
<thead>
<tr>
<th>Sex and Diagnosis</th>
<th>Comorbidity Presence</th>
<th>Basal Activity (pmol/min/µg)</th>
<th>Maximaly-Stimulated Activity (pmol/min/µg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP-I High Ca(^{2+})</td>
<td>Yes</td>
<td>N</td>
<td>8</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>N</td>
<td>8</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>N</td>
<td>16</td>
<td>0.18</td>
</tr>
<tr>
<td>BP-I Norm Ca(^{2+})</td>
<td>Yes</td>
<td>N</td>
<td>9</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>N</td>
<td>6</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>N</td>
<td>15</td>
<td>0.18</td>
</tr>
<tr>
<td>Total</td>
<td>Yes</td>
<td>N</td>
<td>17</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>N</td>
<td>14</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>N</td>
<td>31</td>
<td>0.18</td>
</tr>
</tbody>
</table>
comorbidity type. That is, the patients were divided into those with a comorbid diagnosis of an anxiety disorder and those with a comorbid diagnosis of substance abuse (see Table 7). A univariate ANOVA with maximally-stimulated PKA activity as the dependent variable, and diagnosis, sex and comorbidity type as factors showed a significant main effect of comorbidity type ($F=8.036, df=1.14, p<0.05$). An examination of the means revealed that the patients with a comorbid diagnosis of an anxiety disorder showed significantly higher maximally-stimulated PKA activities than the patients with a comorbid diagnosis of substance abuse ($3.63 \pm 0.85$ and $2.83 \pm 0.56$ pmol/min/µg protein, respectively). The patients with a comorbid diagnosis of an anxiety disorder, however, did not show significantly different maximally-stimulated PKA activity compared with healthy subjects ($t=0.642, df=19, p=0.528$); patients with a comorbid diagnosis of substance abuse also did not show significantly different maximally-stimulated activities compared with the healthy subjects ($t=-1.350, df=22.72, p=0.190$). There were no significant differences in the basal activities or EC50s in the BD patient group stratified by type of comorbidity (see Table 7).

The correlations between subject age, duration of freezer storage and the dependent variables measured in the assays (basal PKA activity, maximally-stimulated PKA activity and EC50 for cAMP activation of PKA) were explored to rule out the possibility that these uncontrolled variables might have confounded the experimental results. There were no significant correlations between subject age, duration of storage at -70°C, basal PKA activity or the EC50 for cAMP activation of PKA. As shown in Figure 15, there was a modest but significant negative correlation between the maximally-stimulated PKA activity and freezer storage time ($r=-0.340, p<0.05, n=48$). Given this finding, a univariate
Table 7- BLCL cytosolic fraction PKA activity in BP-I patients with a lifetime history of psychiatric comorbidity of either an anxiety disorder or substance abuse

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Comorbidity Type</th>
<th>Basal Activity (pmol/min/μg)</th>
<th>Maximaly-Stimulated Activity (pmol/min/μg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>BP-I High Ca²⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety Disorder</td>
<td>3</td>
<td>0.19</td>
<td>0.10</td>
<td>3</td>
</tr>
<tr>
<td>Substance Abuse</td>
<td>5</td>
<td>0.18</td>
<td>0.10</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>0.18</td>
<td>0.09</td>
<td>8</td>
</tr>
<tr>
<td>BP-I Norm Ca²⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety Disorder</td>
<td>2</td>
<td>0.18</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Substance Abuse</td>
<td>7</td>
<td>0.20</td>
<td>0.13</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>0.19</td>
<td>0.10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety Disorder</td>
<td>5</td>
<td>0.18</td>
<td>0.06</td>
<td>5</td>
</tr>
<tr>
<td>Substance Abuse</td>
<td>12</td>
<td>0.19</td>
<td>0.11</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>0.19</td>
<td>0.10</td>
<td>18</td>
</tr>
</tbody>
</table>

*Maximally-stimulated PKA activity was significantly higher in BP-I patients with Anxiety Disorders compared to those with lifetime comorbid Substance Abuse Disorders (F=8.036, df=1,14, p<0.05)
Figure 15: Relationship of freezer storage and maximally-stimulated PKA activity. A statistically significant correlation \((r=0.340, p<0.05)\) was substantially influenced by the two outlier values (A and B) with the longest freezer storage times greater than twice that of the other samples. Reanalyses excluding these outliers showed no significant correlation \((r=0.197, p=0.126)\).
ANCOVA was conducted which did reveal a significant main effect of diagnosis (F=0.649, df=2,44, p=0.528). The relationship identified is likely due to the very long storage time in the freezer for the BLCLs of two healthy control subjects (Case A: 36 months, Case B: 49 months) which both showed low maximally-stimulated PKA activities. The length of freezing for Case A and Case B were greater than two SDs above the mean length of freezing (12.81 ± 7.14 months) and were thus defined as outliers for this variable. With exclusion of these outlier cases there was no significant correlation of maximally-stimulated PKA activity with duration of storage (r=-0.197, p=0.189, n=46) supporting the lack of a significant effect of this variable on PKA activity at least up to 20 months.

There was a significant negative correlation between intracellular [Ca²⁺]₀ and EC50 for cAMP activation of PKA (r=-0.356, p=0.036, n=35). Figure 16 shows the distribution of EC50 and intracellular [Ca²⁺]₀. Given this finding, a univariate ANCOVA was conducted which did not reveal a significant main effect of diagnosis (F=0.024, df=2,31, p=0.976). The relationship identified appears to be due to the low EC50 values for two BP-I High Ca²⁺ subjects (Case A: 14.76 nM, Case B: 14.57 nM). The EC50 values for Case A and Case B were more than two SDs below the mean EC50 for healthy subjects (30.5 ± 3.89 nM) suggesting that they were outliers. Excluding the values for these subjects from the analysis revealed that the EC50 estimates no longer significantly correlated with intracellular [Ca²⁺]₀ (r=-0.155, p=0.389, n=33).

### III.3.2 Comparison of BLCL PKA Activity in MDD, BP-II and Healthy Subjects

Table 8 presents the basal PKA activity, maximally-stimulated ([cAMP]=500 nM) PKA activity, and EC50 for cAMP activation of PKA in BLCLs from the MDD, BP-II, and
Figure 16: Relationship of intracellular $[\text{Ca}^{2+}]_B$ and EC50 for cAMP activation of PKA. A statistically significant correlation ($r=-0.356$, $p<0.05$) was obtained when all values were included. However, after exclusion of the two outlier EC50 values (>2 SDs below the mean EC50 of the healthy subjects ($24.39 \pm 3.11$), no significant correlation ($r=0.155$, $p=0.39$) was obtained.
Table 8- BLCL cytosolic fraction PKA activity parameters in healthy subjects and MDD or BP-II patients

<table>
<thead>
<tr>
<th>Diagnostic Group</th>
<th>Basal Activity (pmol/min/μg)</th>
<th>Maximally-Stimulated Activity (pmol/min/μg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>0.18</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>MDD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>0.19</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>BP-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>0.14</td>
<td>0.08</td>
</tr>
</tbody>
</table>
healthy subjects (n=10 per group) matched on age and sex. The calculated basal PKA activity of one MDD patient was negative and thus excluded from the analysis. ANOVA did not reveal a significant effect of diagnosis (F=0.186, df=2,23, p=0.831) or sex (F=1.019, df=1,23, p=0.323) nor an interaction between these factors (F=1.288, df=2,23, p=0.295) for basal PKA activity between the three comparison groups. Similarly, there were no significant effects of diagnosis (F=0.358, df=2,24, p=0.703), sex (F=2.609, df=1,24, p=0.119), nor an interaction of these factors (F=0.384, df=2,24, p=0.685) for the maximally-stimulated PKA activity between the three comparison groups. Finally, ANOVA for the EC50s for cAMP activation of PKA amongst the three comparison groups did not reveal a significant effect of diagnosis (F=1.998, df=2,24, p=0.157) or sex (F=0.053, df=1,24, p=0.820), nor an interaction of diagnosis and sex (F=1.439, df=2,24, p=0.257).

Tables 9, 10 and 11 show the data for the MDD and BP-II patients stratified by state, lifetime history of a comorbid diagnosis and comorbidity type, respectively. ANOVA of maximally-stimulated PKA activity as the dependent variable and diagnosis, sex and state as factors revealed a significant interaction of sex and state (F=4.752, df=1,13, p<0.05). Post hoc tests revealed significantly lower maximally-stimulated PKA activity in euthymic male patients (2.13 ± 0.69 pmol/min/μg protein) compared with those patients who were ill (2.80 ± 0.49 pmol/min/μg protein) (F=5.67, df=1,13, p<0.05). Furthermore, the comparisons revealed that the female patients who were in an active state
Table 9 - Effect of state of illness on BLCL cytosolic fraction PKA activity from MDD and BP-II patients

<table>
<thead>
<tr>
<th>Sex and Diagnosis</th>
<th>State</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDD</td>
<td>Illness State</td>
<td>6</td>
<td>0.16</td>
<td>0.08</td>
<td>7</td>
<td>2.54</td>
<td>0.65</td>
<td>7</td>
<td>28.8</td>
<td>5.53</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>3</td>
<td>0.13</td>
<td>0.05</td>
<td>3</td>
<td>2.10</td>
<td>0.30</td>
<td>3</td>
<td>30.8</td>
<td>11.23</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
<td>0.15</td>
<td>0.06</td>
<td>10</td>
<td>2.40</td>
<td>0.59</td>
<td>10</td>
<td>29.4</td>
<td>7.03</td>
</tr>
<tr>
<td>BP-II</td>
<td>Illness State</td>
<td>4</td>
<td>0.15</td>
<td>0.11</td>
<td>4</td>
<td>2.34</td>
<td>0.33</td>
<td>4</td>
<td>30.8</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>6</td>
<td>0.14</td>
<td>0.05</td>
<td>6</td>
<td>2.30</td>
<td>0.63</td>
<td>6</td>
<td>32.9</td>
<td>6.04</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>10</td>
<td>0.14</td>
<td>0.08</td>
<td>10</td>
<td>2.31</td>
<td>0.50</td>
<td>10</td>
<td>32.1</td>
<td>4.90</td>
</tr>
<tr>
<td>Total Female</td>
<td>Illness State</td>
<td>4</td>
<td>0.18</td>
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<td>2.06</td>
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<tr>
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<td>Euthymic</td>
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<td>0.13</td>
<td>0.04</td>
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<td>2.36</td>
<td>0.24</td>
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<td>35.3</td>
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</tr>
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<td>0.05</td>
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<td>9</td>
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</tr>
<tr>
<td>Male</td>
<td>Illness State</td>
<td>6</td>
<td>0.14</td>
<td>0.10</td>
<td>6</td>
<td>2.80</td>
<td>0.49</td>
<td>6</td>
<td>30.8</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>5</td>
<td>0.14</td>
<td>0.06</td>
<td>5</td>
<td>2.13</td>
<td>0.69</td>
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<td>8.46</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>0.14</td>
<td>0.08</td>
<td>11</td>
<td>2.49</td>
<td>0.66</td>
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</tr>
<tr>
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<td>Illness State</td>
<td>10</td>
<td>0.15</td>
<td>0.09</td>
<td>11</td>
<td>2.46</td>
<td>0.54</td>
<td>11</td>
<td>29.5</td>
<td>4.65</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>9</td>
<td>0.14</td>
<td>0.05</td>
<td>9</td>
<td>2.24</td>
<td>0.53</td>
<td>9</td>
<td>32.2</td>
<td>7.44</td>
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<td>Total</td>
<td>19</td>
<td>0.15</td>
<td>0.08</td>
<td>20</td>
<td>2.36</td>
<td>0.54</td>
<td>20</td>
<td>30.7</td>
<td>6.05</td>
</tr>
</tbody>
</table>

a Maximally-stimulated PKA activity was significantly lower in ill female patients compared to ill male patients (F=7.35, df=1,13, p<0.05)

b Maximally-stimulated PKA activity was significantly lower in euthymic male patients compared to ill male patients (F=5.67, df=1,13, p<0.05)
Table 10 - BLCL cytosolic fraction PKA activity in MDD and BP-II patients with and without a lifetime history of psychiatric comorbidity

<table>
<thead>
<tr>
<th>Sex and Diagnosis</th>
<th>Comorbidity Presence</th>
<th>Basal Activity (pmol/min/μg)</th>
<th>Maximally-Stimulated Activity (pmol/min/μg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>MDD</td>
<td>Yes</td>
<td>3</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>6</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>BP-II</td>
<td>Yes</td>
<td>6</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>4</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>10</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td>Total</td>
<td>Yes</td>
<td>9</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>10</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>19</td>
<td>0.15</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Table 11 - BLCL cytosolic fraction PKA activity in MDD and BP-II patients with a lifetime history of psychiatric comorbidity of either anxiety disorder or substance abuse

<table>
<thead>
<tr>
<th>Sex and Diagnosis</th>
<th>Comorbidity Type</th>
<th>Basal Activity (pmol/min/µg)</th>
<th>Maximal-Stimulated Activity (pmol/min/µg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>MDD</td>
<td>Anxiety Disorder</td>
<td>2</td>
<td>0.14</td>
<td>-</td>
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<tr>
<td></td>
<td>Substance Abuse</td>
<td>1</td>
<td>0.11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>BP-II</td>
<td>Anxiety Disorder</td>
<td>3</td>
<td>0.18</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>3</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>6</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>Anxiety Disorder</td>
<td>5</td>
<td>0.16</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>4</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
<td>0.14</td>
<td>0.08</td>
</tr>
</tbody>
</table>
of illness at the time of the study showed significantly lower maximally-stimulated PKA activity (2.06 ± 0.26 pmol/min/µg protein) compared with the ill male patients (2.80 ± 0.49 pmol/min/µg protein) (F=7.35, df=1,13, p<0.05). There were no significant differences for the basal PKA activity or the EC50 for cAMP activation of PKA among the patients stratified by the state of illness (see Table 9).

Stratifying the MDD and BP-II patients by the presence of a comorbid diagnosis, or by comorbiditiy type did not reveal any significant differences for either the basal PKA activity, maximally-stimulated PKA activity or the EC50 for cAMP activation of PKA (see Tables 10 and 11). There were also no significant correlations between age, intracellular [Ca^{2+}]_i or duration of time that cells were stored in the freezer before assay and basal PKA activity, maximally-stimulated PKA activity or the EC50 for cAMP activation of PKA.

III. 3.3 Comparison of BP-I High Ca^{2+} and BP-I Normal Ca^{2+} Patients with MDD and BP-II Patients

There were no significant differences found for the dependent variables of basal PKA activity, maximally-stimulated PKA activity or EC50 for cAMP activation of PKA between the patient groups and the healthy subjects in either of the first two analyses (BP-I High Ca^{2+}, BP-I Normal Ca^{2+} and healthy subjects or MDD, BP-II and healthy subjects). It was of interest, however, to compare the measures of the four patient groups in one analysis to determine if there were any important differences amongst the diagnostic groups.
As mentioned previously, seven of the BLCLs from the healthy subjects used in the first analysis (first batch: BP-I High Ca$^{2+}$ and BP-I Normal Ca$^{2+}$ comparison) were also used in the second analysis (second batch: MDD and BP-II comparison). A comparison of the values of the dependent variables for those seven healthy subjects between the first batch and the second batch, while not statistically significant, showed a trend for lower basal PKA activity in the second batch (0.15 ± 0.05 pmol/min/μg protein) compared with the first batch (0.20 ± 0.09 pmol/min/μg protein) (t=2.286, df=6, p=0.06). Similarly, the maximally-stimulated PKA activity for the second batch (2.26 ± 0.46 pmol/min/μg protein) also tended to be lower compared with the first batch (3.13 ± 0.95 pmol/min/μg protein) (t=2.087, df=6, p=0.08). The EC50 for cAMP activation of PKA was not significantly different between the two batches (t=1.093, df=4, p=0.336). The differences in basal and maximally-stimulated PKA activity between the two batches could not be attributed to a storage effect or to any effect of reagent stability or reagent batch effect. As such, the observed batch effect was attributed to an extraneous variable source, likely assay related.

To allow some comparison of PKA activity data from MDD and BP-II with BP-I patients, data from the former group were adjusted for the interbatch assay effect with calculated adjustment factors. The adjustment factors for each of the three dependent variables were calculated as the ratio of the mean maximally-stimulated PKA activities for all healthy subjects from the two batches (3.28/2.54=1.29), the ratio of the mean basal PKA activities for all healthy subjects from the two batches (0.16/0.15=1.08) and the ratio of the mean EC50 for cAMP activation of PKA for all healthy subjects from the two batches (30.49/27.05=1.13). Therefore, the maximally-stimulated activities of the MDD and BP-II patients were adjusted by a factor of 1.29, the basal activities by a factor of 1.08 and the
EC50s by a factor of 1.13. The adjusted dependent variables for the MDD and BP-II patients were then compared with the BP-I High Ca\(^{2+}\) and BP-I Normal Ca\(^{2+}\) patients.

Table 12 shows the basal PKA activity, maximally-stimulated ([cAMP]=500 nM) PKA activity, and EC50 for cAMP activation of PKA in BLCLs from the BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), MDD and BP-II patients. ANOVA for basal PKA activity did not reveal significant effects of diagnosis (F=0.223, df=3,42, p=0.880), or sex (F=0.037, df=1,42, p=0.848), or an interaction of the two factors (F=0.617, df=3,42, p=0.608). Similarly, ANOVA for maximally-stimulated PKA activity did not show significant effects of diagnosis (F=0.168, df=3,44, p=0.917), sex (F=0.033, df=1,44, p=0.857), nor an interaction of these factors (F=1.371, df=3,44, p=0.264).

ANOVA for the EC50 for cAMP activation of PKA showed a significant effect of diagnosis (F=4.138, df=3,35, p<0.05) but not sex (F=0.011, df=1,35, p=0.918) nor an interaction of diagnosis and sex (F=1.147, df=3,35, p=0.344). Tukey post hoc comparison revealed that the effect of diagnosis was attributable to higher EC50 values in BP-II patients (36.24 ± 5.54 nM) compared with BP-I High Ca\(^{2+}\) patients (28.05 ± 5.64 nM) (29%, p<0.05). The Hill slopes were next compared to assess the functional relevance of this small difference in EC50 values. The Hill slope reflects the interaction of two molecules of cAMP and the R-subunits of PKA which are known to show positive cooperativity. As shown in Figure 17, almost all of the concentration-response curves generated by the assays showed Hill slopes which were >1 reflecting the positive cooperativity (mean Hill slope is 1.45 ± 0.29, n=65). Comparison of the Hill slopes for cAMP-stimulated PKA activity in cytosolic BLCL fractions between the BP-II
### Table 12 - BLCL cytosolic fraction PKA activity parameters in BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), MDD and BP-II patients adjusted for batch effects

<table>
<thead>
<tr>
<th>Diagnostic Group</th>
<th>Basal Activity (pmol/min/μg)</th>
<th>Maximal-Stimulated Activity (pmol/min/μg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>BP-I High Ca(^{2+})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>0.19</td>
<td>0.11</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>0.18</td>
<td>0.10</td>
</tr>
<tr>
<td>BP-I Norm Ca(^{2+})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>MDD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>0.20</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>BP-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
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<td>0.04</td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>0.16</td>
<td>0.10</td>
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<td>Total</td>
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<td>0.09</td>
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<td>Total</td>
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<tr>
<td>Female</td>
<td>24</td>
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<td>0.08</td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
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<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>0.16</td>
<td>0.09</td>
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</tbody>
</table>

* EC50 for cAMP activation of PKA was significantly higher in BP-II patients compared to BP-I High Ca\(^{2+}\) patients (F=4.138, df=3,35, p<0.05)
Figure 17: Hill slopes for concentration-response relationships of PKA activity for cAMP in cytosolic fraction of BLCLs from all subjects. The mean of the Hill slopes was 1.45 ± 0.29 (N=65).
patients and the BP-I High Ca\(^{2+}\) patients (see Figure 18), however, revealed no statistically significant difference \(t=0.315, df=20, p=0.756\).

Stratifying the patient groups by state or the presence of a comorbid diagnosis did not reveal any statistically significant differences between the four patient groups for basal PKA activity, maximally-stimulated PKA activity, or EC50 for cAMP activation of PKA (see Tables 13 and 14). ANOVA with maximally-stimulated PKA activity as the dependent variable and diagnosis, sex and comorbidity type as factors revealed a significant effect of comorbidity type \(F=6.338, df=1,21, p<0.05\). Patients with a comorbid diagnosis of an anxiety disorder showed significantly greater maximally-stimulated PKA activity compared with patients with a comorbid diagnosis of substance abuse \((3.34 \pm 0.71 \text{ and } 2.81 \pm 0.58 \text{ pmol/min/\text{g protein, respectively}) (See Table 15.}

III. 3.4 Age of Onset

A variety of observations support the idea that age of onset may reflect additional pathophysiological components in the BD syndrome. Age of onset, defined here based on historical recall of first definite signs and symptoms of an episode of depression or mania, for BD (BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), BP-II) patients showed a significant correlation with maximally-stimulated PKA activity \(r=0.516, p<0.01, n=33\). The relationship was also significant \(r=0.408, p<0.05, n=26\) if only the BP-I patients (BP-I High Ca\(^{2+}\) and BP-I Normal Ca\(^{2+}\)) were included in the analysis. Figure 19 presents a scatter diagram highlighting the relationship between the two variables, revealing that the greater the age of onset, the higher the maximally-stimulated PKA activity attained.
Figure 18: Comparison of Hill slopes between BP-I High Ca\textsuperscript{2+} and BP-II patients. The Hill slopes were not significantly different between the two diagnostic groups ($t=0.315$, df=20, $p=0.76$).
Table 13 – Effect of state of illness on BLCL cytosolic fraction PKA activity from BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), MDD and BP-II patients adjusted for batch effects

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>State</th>
<th>Basal Activity (pmol/min/µg)</th>
<th>Maximaly-Stimulated Activity (pmol/min/µg)</th>
<th>EC50 ([cAMP] nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>BP-I High Ca(^{2+})</td>
<td>Ill</td>
<td>5</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>11</td>
<td>0.20</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>0.18</td>
<td>0.10</td>
</tr>
<tr>
<td>BP-I Normal Ca(^{2+})</td>
<td>Ill</td>
<td>7</td>
<td>0.19</td>
<td>0.13</td>
</tr>
<tr>
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<td>Euthymic</td>
<td>8</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>15</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>MDD</td>
<td>Ill</td>
<td>6</td>
<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>3</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>BP-II</td>
<td>Ill</td>
<td>4</td>
<td>0.16</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Euthymic</td>
<td>6</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>10</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>Illness State</td>
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<td>0.10</td>
</tr>
<tr>
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<td>Euthymic</td>
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<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>0.16</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Table 14 – BLCL cytosolic fraction PKA activity in BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), MDD and BP-II patients adjusted for batch effects with and without a lifetime history of psychiatric comorbidity

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Comorbidity</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP-I High Ca(^{2+})</td>
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<td>8</td>
<td>0.18</td>
<td>0.09</td>
<td>8</td>
<td>2.81</td>
<td>0.46</td>
<td>6</td>
<td>26.7</td>
<td>7.30</td>
</tr>
<tr>
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<td>No</td>
<td>8</td>
<td>0.18</td>
<td>0.10</td>
<td>8</td>
<td>3.16</td>
<td>0.68</td>
<td>6</td>
<td>29.4</td>
<td>3.53</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>0.18</td>
<td>0.10</td>
<td>16</td>
<td>2.99</td>
<td>0.59</td>
<td>12</td>
<td>28.1</td>
<td>5.64</td>
</tr>
<tr>
<td>BP-I Normal Ca(^{2+})</td>
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<td>0.10</td>
<td>10</td>
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<td>30.0</td>
<td>2.69</td>
</tr>
<tr>
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<td>No</td>
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<td>0.08</td>
<td>6</td>
<td>2.95</td>
<td>0.63</td>
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<td>33.6</td>
<td>3.78</td>
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<tr>
<td></td>
<td>Total</td>
<td>15</td>
<td>0.18</td>
<td>0.09</td>
<td>16</td>
<td>3.13</td>
<td>0.76</td>
<td>11</td>
<td>31.3</td>
<td>3.45</td>
</tr>
<tr>
<td>MDD</td>
<td>Yes</td>
<td>3</td>
<td>0.14</td>
<td>0.01</td>
<td>3</td>
<td>2.90</td>
<td>0.60</td>
<td>3</td>
<td>32.0</td>
<td>8.34</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>6</td>
<td>0.18</td>
<td>0.09</td>
<td>7</td>
<td>3.19</td>
<td>0.85</td>
<td>7</td>
<td>33.7</td>
<td>8.38</td>
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<td></td>
<td>Total</td>
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<td>0.16</td>
<td>0.08</td>
<td>10</td>
<td>3.10</td>
<td>0.76</td>
<td>10</td>
<td>33.2</td>
<td>7.94</td>
</tr>
<tr>
<td>BP-II</td>
<td>Yes</td>
<td>6</td>
<td>0.16</td>
<td>0.10</td>
<td>6</td>
<td>2.91</td>
<td>0.64</td>
<td>6</td>
<td>36.3</td>
<td>6.76</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>4</td>
<td>0.14</td>
<td>0.06</td>
<td>4</td>
<td>3.09</td>
<td>0.74</td>
<td>4</td>
<td>36.2</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>0.15</td>
<td>0.09</td>
<td>10</td>
<td>2.99</td>
<td>0.64</td>
<td>10</td>
<td>36.2</td>
<td>5.54</td>
</tr>
<tr>
<td>Total</td>
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<td>26</td>
<td>0.18</td>
<td>0.09</td>
<td>27</td>
<td>3.00</td>
<td>0.68</td>
<td>22</td>
<td>31.1</td>
<td>6.78</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>24</td>
<td>0.16</td>
<td>0.09</td>
<td>25</td>
<td>3.10</td>
<td>0.69</td>
<td>21</td>
<td>32.9</td>
<td>5.90</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>0.16</td>
<td>0.09</td>
<td>52</td>
<td>3.05</td>
<td>0.68</td>
<td>43</td>
<td>32.0</td>
<td>6.36</td>
</tr>
</tbody>
</table>
Table 15 – BLCL cytosolic fraction PKA activity in BP-I High Ca\(^{2+}\), BP-I Normal Ca\(^{2+}\), MDD and BP-II patients adjusted for batch effects with a lifetime history of psychiatric comorbidity of either an anxiety disorder or substance abuse

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Comorbidity Type</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
</tr>
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<tbody>
<tr>
<td><strong>BP-I High Ca(^{2+})</strong></td>
<td>Anxiety Disorder</td>
<td>3</td>
<td>0.19</td>
<td>0.10</td>
<td>3</td>
<td>3.14</td>
<td>0.20</td>
<td>2</td>
<td>24.0</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>5</td>
<td>0.18</td>
<td>0.10</td>
<td>5</td>
<td>2.63</td>
<td>0.49</td>
<td>4</td>
<td>28.1</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8</td>
<td>0.18</td>
<td>0.09</td>
<td>8</td>
<td>2.81</td>
<td>0.46</td>
<td>6</td>
<td>26.7</td>
<td>7.30</td>
</tr>
<tr>
<td><strong>BP-I Normal Ca(^{2+})</strong></td>
<td>Anxiety Disorder</td>
<td>2</td>
<td>0.18</td>
<td>0.03</td>
<td>2</td>
<td>4.36</td>
<td>0.99</td>
<td>2</td>
<td>32.0</td>
<td>3.19</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>7</td>
<td>0.20</td>
<td>0.13</td>
<td>8</td>
<td>2.96</td>
<td>0.60</td>
<td>5</td>
<td>29.2</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
<td>0.19</td>
<td>0.10</td>
<td>10</td>
<td>3.24</td>
<td>0.86</td>
<td>7</td>
<td>30.0</td>
<td>2.69</td>
</tr>
<tr>
<td><strong>MDD</strong></td>
<td>Anxiety Disorder</td>
<td>2</td>
<td>0.14</td>
<td>0.01</td>
<td>2</td>
<td>2.84</td>
<td>0.83</td>
<td>2</td>
<td>27.5</td>
<td>4.09</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>1</td>
<td>0.13</td>
<td>-</td>
<td>1</td>
<td>3.01</td>
<td>-</td>
<td>1</td>
<td>41.0</td>
<td>-</td>
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<td>Total</td>
<td>3</td>
<td>0.14</td>
<td>0.01</td>
<td>3</td>
<td>2.90</td>
<td>0.60</td>
<td>3</td>
<td>32.0</td>
<td>8.34</td>
</tr>
<tr>
<td><strong>BP-II</strong></td>
<td>Anxiety Disorder</td>
<td>3</td>
<td>0.19</td>
<td>0.13</td>
<td>3</td>
<td>3.18</td>
<td>0.24</td>
<td>3</td>
<td>39.7</td>
<td>7.21</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>3</td>
<td>0.14</td>
<td>0.08</td>
<td>3</td>
<td>2.65</td>
<td>0.86</td>
<td>3</td>
<td>32.8</td>
<td>5.15</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>6</td>
<td>0.16</td>
<td>0.10</td>
<td>6</td>
<td>2.91</td>
<td>0.64</td>
<td>6</td>
<td>36.3</td>
<td>6.76</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>Anxiety Disorder</td>
<td>10</td>
<td>0.18</td>
<td>0.09</td>
<td>10</td>
<td>3.34</td>
<td>0.71</td>
<td>9</td>
<td>31.8</td>
<td>8.23</td>
</tr>
<tr>
<td></td>
<td>Substance Abuse</td>
<td>16</td>
<td>0.18</td>
<td>0.10</td>
<td>17</td>
<td>2.81</td>
<td>0.58</td>
<td>13</td>
<td>30.6</td>
<td>5.89</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>26</td>
<td>0.18</td>
<td>0.09</td>
<td>27</td>
<td>3.00</td>
<td>0.68</td>
<td>22</td>
<td>31.1</td>
<td>6.78</td>
</tr>
</tbody>
</table>

*Maximally-stimulated PKA activity is significantly higher in patients with a comorbid diagnosis of Anxiety Disorder compared to patients with a comorbid diagnosis of Substance Abuse \((F=6.338, df=1,21, p<0.05)\)*
Figure 19: Relationship of age of onset and maximally-stimulated PKA activity. The regression line (---) (r=0.52, p<0.01) and 95% confidence intervals (…) of the data are shown.
The relationship between age of onset and maximally-stimulated PKA activity was further analyzed as a categorical variable by subdividing age of onset into two main subgroups: early onset (≤19 years) and adult onset (≥20 years) (see Table 16). Previous meta-analyses of age of onset studies support the construct validity of such a subdivision (Goodwin & Jamison, 1990). Analysis of age of onset in this manner revealed that the early onset BD patients had significantly lower (18%) maximally-stimulated activities (2.75 ± 0.59 pmol/min/μg protein) compared with the adult onset BD patients (3.36 ± 0.73 pmol/min/μg protein) (t=-2.664, df=31, p<0.05) (see Table 16). When only the BP-I (BP-I High Ca²⁺, BP-I Normal Ca²⁺) patients were included in the analysis, the early onset BP-I patients also showed a trend toward lower (16%) maximally-stimulated PKA activities (2.79 ± 0.61 pmol/min/μg protein) compared with the adult onset BP-I patients (3.33 ± 0.76 pmol/min/μg protein) (t=-1.968, df=24, p=0.061).

III.3.5 Analysis of PKA Activity as Percent of Basal Activity

Maximally-stimulated PKA activity was approximately 20-fold that of the basal PKA activity. In addition, the standard deviation of the basal PKA activity was greater than 50% of the mean basal PKA activity for the healthy controls (0.16 ± 0.09). Analyses of the data in which the maximally-stimulated PKA activity and EC50 for cAMP activation of PKA were expressed as the percent of basal activity did not reveal any statistically significant findings, however. This was not unexpected considering the small magnitude of the basal activity.
Table 16 – BLCL cytosolic fraction maximally-stimulated PKA activity in BP-I High Ca\textsuperscript{2+}, BP-I Normal Ca\textsuperscript{2+} and BP-II patients (adjusted for batch effects) stratified by Onset Factor (early onset (≤19 years) or adult onset (≥20 years))

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Onset Factor</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP-I High Ca\textsuperscript{2+}</td>
<td>Early onset</td>
<td>10</td>
<td>13.10</td>
<td>3.35</td>
<td>10</td>
<td>2.80</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Adult onset</td>
<td>4</td>
<td>21.50</td>
<td>3.32</td>
<td>4</td>
<td>3.41</td>
<td>0.31</td>
</tr>
<tr>
<td>BP-I Normal Ca\textsuperscript{2+}</td>
<td>Early onset</td>
<td>3</td>
<td>13.00</td>
<td>4.36</td>
<td>3</td>
<td>2.75</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Adult onset</td>
<td>9</td>
<td>22.00</td>
<td>2.00</td>
<td>9</td>
<td>3.28</td>
<td>0.91</td>
</tr>
<tr>
<td>BP-II</td>
<td>Early onset</td>
<td>4</td>
<td>15.00</td>
<td>4.08</td>
<td>4</td>
<td>2.64</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>Adult onset</td>
<td>3</td>
<td>24.33</td>
<td>6.11</td>
<td>4</td>
<td>3.54</td>
<td>0.58</td>
</tr>
<tr>
<td>Total</td>
<td>Early onset</td>
<td>17</td>
<td>13.53</td>
<td>3.54</td>
<td>17</td>
<td>2.75\textsuperscript{a}</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>Adult onset</td>
<td>16</td>
<td>22.31</td>
<td>3.22</td>
<td>16</td>
<td>3.36</td>
<td>0.73</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Maximally-stimulated PKA activity was significantly lower in patients with early onset compared to patients with adult onset (t=-2.664, df=31, p<0.05)
CHAPTER IV

DISCUSSION
The main objective of this study was to compare PKA activity in BLCLs from BD patients with healthy subjects to determine if the function of this transducing protein is altered in this disorder in a trait-dependent fashion. A thorough review of the literature did not reveal previous characterization of PKA activity specifically in BLCLs. Thus, a secondary objective was to establish the specificity, sensitivity and reliability of the PKA activity assay in BLCLs. The optimization procedure is thus first addressed, followed by a discussion of the results from the experiments that relate to the main objective of this thesis.

IV.1 Optimization of the PKA Activity Assay

IV.1.1 Method for Determining PKA-Specific Activity

An important part of the optimization procedure was to ascertain an appropriate method for the determination of phosphotransferase activity that is specific to PKA. A comparison of the substrate/inhibitor-cocktail and the PKA inhibitor methods suggested that the latter method allowed for the most accurate measurement of PKA-specific activity. While not statistically significant, preliminary comparisons showed that the PKA inhibitor method gave slightly lower measurable PKA-specific activity than the substrate/inhibitor-cocktail method. This finding offered support for the hypothesis that the substrate/inhibitor-cocktail method might not accurately control for all active kinases and thus over-estimate kinase activity that is specific to PKA. The use of the PKA inhibitor method has been promoted in recent methodological reviews (Murray et al 1995) as a more specific alternative procedure and is used by many groups to define PKA-specific activity (Giembycz & Diamond 1990; Langlands & Rodger 1990; Shelton et al 1996).
One particular kinase that may contribute to the over-estimation of activity specific to PKA is cGMPdk. This kinase, which is activated by cGMP, can phosphorylate Kemptide (Km=231 μM) (Glass et al 1986) and can also be activated by cAMP (Ka=0.3 μM) (Torphy et al 1982) at high nanomolar concentrations. Indeed, it was shown that as the concentration of cAMP increased from the basal (no added cAMP) to the maximally-stimulated (500 nM cAMP) condition, the fraction of PKI-resistant activity increased by 30-50%. This suggested that a portion of the total activity, as measured by the formation of \(^{33}\)P-Kemptide, was activated by cAMP and resistant to the PKA-specific inhibitor. To deduce whether this non-PKA kinase activity was attributable to cGMPdk, the effect of the cGMPdk-specific inhibitor KT5823 (1-2 μM) was explored. In the BLCL preparation used here, however, KT5823 used in concert with PKI(5-22)amide did not reduce the activity resistant to the latter inhibitor. Higher concentrations of KT5823 are known to inhibit PKA (Ki >10 μM) (Kase et al 1987) and, thus, were not explored.

Given the failure of the cGMPdk-inhibitor to decrease the amount of PKI(5-22)amide-resistant activity, it was difficult to draw definitive conclusions about the kinase(s) responsible for the activity. It is possible that cGMPdk does not contribute to the inhibitor-resistant activity in BLCLs, thereby explaining the ineffectiveness of the cGMPdk-inhibitor to decrease the PKA-inhibitor-resistant activity. Alternatively, the cGMPdk-inhibitor, KT5823, might be rendered inactive by some component of the reagent mixture. Another possibility is that the concentrations of KT5823 that were employed (1-2 μM) were insufficient to inhibit cGMPdk, although this is unlikely due to the low Ki of the inhibitor (234 nM) (Kase et al 1987). Torphy et al (1982) were able to show in canine trachealis homogenates that the PKI-resistant portion of activity was similarly increased by
both cAMP and cGMP, suggesting that the enzyme responsible for the activity in that preparation was cGMPdk. The authors suggested that overlooking the contribution of cGMPdk when measuring PKA activity could result in an over-estimation of total PKA-specific activity (Torphy et al 1982).

Alternatively, the kinase that is being activated by cAMP and resistant to PKI(5-22)amide might be one that is stimulated indirectly through the Epac-Rap1 pathway. It is now well established that cAMP, in addition to stimulating PKA, also binds and activates Epac, a GEF which activates Rap1, and consequently a signal transduction cascade involving various kinases including MEK and MAPK (de Rooij et al 1998; Roberson et al 1999). Thus, it is possible that an unknown kinase activated by the Epac-mediated pathway might contribute to the activity that is resistant to the PKA inhibitor. A more in depth examination of the PKI(5-22)amide-resistant activity in BLCLs is clearly needed to ascertain the particular kinases involved.

The stimulation of PKI(5-22)amide-resistant and, as yet, unidentified kinases in BLCLs by cAMP, highlights the importance of using the PKA inhibitor method for the determination of PKA-specific activity, as suggested by Torphy et al (1982). PKI(5-22)amide was used at a concentration of 5 μM in the assays in this study. Increasing the concentration of inhibitor to 10 μM did not produce any further increase in PKA-specific activity, indicating that a concentration of 5 μM was saturating for the BLCL preparation. A methodological review of PKA inhibitors (Kemp et al 1988) advised the use of concentrations equal to, or less than 5 μM for the determination of PKA-specific activity.
This concentration of inhibitor did not inhibit a variety of other kinases, including cGMPdK (Kemp et al 1988), thus supporting the choice of concentration used in this study.

PKI(5-22amide) (5 μM) inhibited approximately 40% of basal PKA activity and 80% of maximally-stimulated PKA activity in the BLCL preparation. This observation concurs with the percent of activity that is inhibited by PKA inhibitors found in reports on other tissues. For example, in canine trachealis homogenates, PKI was shown to inhibit 70% of cAMP (5 μM)-stimulated activity (Torphy et al 1982). Similarly, in intact human platelets, 80% of cAMP (5 μM)-stimulated activity was inhibited by the PKA inhibitor IP20 (Hatmi et al 1996). Of note, in some assay preparations in which purified C-subunits were used, PKA-specific inhibitors were shown to inhibit 100% of cAMP-stimulated activity suggesting that the activity which is PKI-resistant is not PKA-specific (Cheng et al 1986; Glass et al 1992). Furthermore, in soluble fractions of homogenized guinea-pig tracheal smooth muscle, a PKA inhibitor was shown to inhibit 100% of cAMP-stimulated activity suggesting that the portion of inhibitable activity may be tissue-specific (Langlands & Rodger 1990).

In conclusion, the definition of PKA activity by the use of PKI(5-22)amide appears to offer greater specificity than afforded by the substrate/inhibitor-cocktail method for the determination of PKA-specific activity in the BLCL preparation. Further characterization of the nature of the kinase(s) contributing to the non-PKA activity in the BLCLs would provide direction on how to suppress these non-specific contributions to assays of PKA activity.
IV.1.2 Cytosolic and Particulate Fractions – Centrifugal Force

A series of experiments were conducted to confirm the choice of centrifugal force to fractionate the BLCL homogenates, as well as to determine the fraction(s) (cytosolic or particulate) in which activity would be measured in subsequent experiments. It was shown that when a centrifugal force of 48,000 g was used to fractionate BLCL homogenates, cAMP (1 μM)-stimulated PKA activity in the particulate fraction was approximately 30% of that in the respective cytosolic fraction, and the activity did not show a concentration-response relationship for cAMP stimulation. Attempts to increase the amount of measurable activity in the particulate fraction by increasing the centrifugal force to 100,000 g were unsuccessful. The increased centrifugal force resulted in approximately 40% less PKA activity in the cytosolic fraction without a corresponding increase in activity in the particulate fraction. These findings suggest that the greater centrifugal force (100,000 g) may disrupt the PKA enzyme rendering it inactive. Furthermore, the results suggest that the majority of PKA activity in BLCLs is associated with the cytosolic fraction, and not the particulate fraction, when homogenates are fractionated at either 48,000 or 100,000 g.

Given the small amount of measurable particulate PKA activity found with fractionation at 48,000 g or 100,000 g, and the negative effect of the 100,000 g force on the cytosolic fraction activity, the weaker force was chosen for cytosolic preparation for subsequent assays. Furthermore, only the resulting cytosolic fractions were used for PKA activity assays because of the negligible activity in the particulate fractions.

Other investigators reporting on PKA activity in peripheral tissues have similarly shown lower activity in the particulate as compared with the cytosolic fractions when
centrifugal forces of 48,000 g or lower were used to fractionate the tissue (Torphy et al 1982; Giembycz & Diamond 1990; Langlands & Rodger 1990; Manier et al 1997; Shelton et al 1996). For example, in canine trachealis smooth muscle fractionated with a force of 8,000 g (Torphy et al 1982) and in guinea pig trachealis smooth muscle fractionated with a force of 23,000 g (Langlands & Rodger 1990), the particulate fractions showed maximally-stimulated PKA activity that was only 20% of that in the cytosolic fraction. Thus, the results obtained here in BLCLs agree with earlier findings of low amounts of particulate fraction PKA activity in peripheral cells.

Previous studies that have examined the tissue distribution of PKA also provide support for the finding of very low PKA activity in the particulate fraction of BLCLs. In rabbit skeletal muscle, heart, kidney and liver nearly all the PKA enzyme was shown to partition with the cytosolic fraction when forces of 10,000 g were used to fractionate the tissue. By contrast, the enzyme was more evenly distributed in rat brain with 60% of the enzyme in the cytosolic fraction and 40% in the particulate fraction (Hofmann et al 1977), suggesting important differences between peripheral cells and brain tissue. Indeed, in homogenates from post-mortem brain of BD patients and healthy subjects fractionated at 48,000 g, the resulting particulate fraction showed maximally-stimulated PKA activity that ranged from less than 50% of that in the cytosolic fraction in temporal cortex to 300% of the PKA activity in the occipital cortex cytosolic fraction (Fields et al 1999). Although PKA activity in particulate fractions from post-mortem brain showed cortical region-specific differences, it is evident that the activity in the particulate fractions from this tissue constitutes a larger percentage (50-300%) of cytosolic fraction PKA activity than in peripheral cells (20%) (Torphy et al 1982; Langlands & Rodger 1996). Therefore, the low
amount of particulate PKA activity in BLCLs found in this study concurs with the results of other studies of PKA activity in peripheral cells and contrasts with brain tissue in which the distribution and activity of the enzyme is more evenly divided between the cytosolic and particulate fractions.

Further inferences may be drawn pertaining to the abundance and particular isoform composition of PKA in BLCLs. The low activity in the particulate fraction may reflect fewer RII subunits that are known to be more prevalent in the particulate fractions through association with AKAPs. The higher PKA activity in the cytosolic fraction, by contrast, suggests larger amounts of RI subunits that are, for the most part, associated with the cytosolic fraction (Deviller et al 1984; Hansson et al 1999). In addition, the findings of similar distributions of activity in BLCLs as in other peripheral tissues, but not cerebral cortex, suggests that these cell lines have low amounts of R- and C- β-subunits that are predominantly expressed in the CNS (Clegg et al 1988) in contrast to the α-subunits which occur ubiquitously in tissues and are expressed in greater amounts relative to the β-subunits (Doskeland et al 1993; Brandon et al 1998).

The possibility should also be entertained that a factor involved in the handling of the BLCLs during freezing, homogenization or centrifugation, might affect the distribution of PKA activity. It is conceivable that some aspect of the methodology might cause the dissociation of the R-subunits and AKAPs thereby releasing PKA-II holoenzyme that might normally associate with the particulate fraction, into the cytosolic fraction. This would result in the artificial association of the majority of PKA activity with the cytosolic fraction. Further experiments exploring the isozyme composition of PKA in BLCLs should
allow more definitive conclusions to be drawn about the distribution of PKA activity between the particulate and cytosolic fractions.

IV.1.3 Concentration-Response Relationship of PKA Activity for cAMP

As presented in the results, PKA activity stimulated by cAMP in cytosolic fractions increased in a concentration-dependent manner reaching a maximal response at \( \approx 500 \) nM cAMP. The concentration-response relationship for stimulation of BLCL cytosolic PKA activity by cAMP was best fit by a typical sigmoidal log concentration-response relationship. The mean basal and maximally-stimulated PKA activity, as well as the EC50 for cAMP activation of PKA in the cytosolic fraction of BLCLs from healthy subjects, were \( 0.16 \pm 0.09, 3.28 \pm 1.14 \) pmol/min/\( \mu \)g protein and \( 30.5 \pm 3.89 \) nM, respectively. These observations are very similar to those reported in fibroblasts in which 700 nM of cAMP was required to reach the maximal PKA activity (Manier et al 1996). The basal and maximally-stimulated PKA activity, and EC50 for cAMP activation of PKA in the cytosolic fraction of the fibroblasts were \( 0.46 \pm 0.05, 2.04 \pm 0.09 \) pmol/min/\( \mu \)g protein and 18 nM cAMP, respectively (Manier et al 1996). It is noteworthy that maximally-stimulated PKA activity and EC50 for cAMP activation of PKA in BLCLs are similar to the values reported for fibroblasts while basal activity in the former tissue is 3-fold lower.

The similar PKA activity values found in BLCLs and fibroblasts can be compared with PKA activity in the cytosolic fraction of temporal cortex of post-mortem brain from non-psychiatric, non-neurological subjects. In post-mortem brain, five-fold higher concentrations of cAMP (2.5 \( \mu \)M) were required to reach maximal stimulation of PKA activity in comparison to BLCLs. Furthermore, in this tissue, basal and maximally-
stimulated PKA activity, and EC50 for cAMP activation of PKA were 0.200 ± 0.08, 2.28 ± 0.80 pmol/min/μg protein and 945 ± 407 nM, respectively. BLCL basal PKA and maximally-stimulated PKA activity in BLCLs is comparable to that found in neuronal tissue. In contrast, the EC50 for cAMP activation of PKA found in BLCLs is approximately 32-fold lower than the EC50 found in post-mortem brain.

The results suggest that PKA in BLCLs shows similar characteristics to PKA in other peripheral cells, but different properties from PKA in brain tissue. The findings further suggest that PKA in BLCLs is more responsive to cAMP than PKA in the brain as lower concentrations of cAMP will activate the enzyme in the BLCLs given the considerably lower EC50 for cAMP activation of PKA in these cells, as compared with that in post-mortem brain. It follows that PKA in BLCLs might be comprised mainly of PKA-I holoenzymes. PKA-I holoenzymes have a higher affinity for cAMP than PKA-II (Amieux et al 1997) and RII subunits have been shown to have a higher affinity for C-subunits than RI subunits (Otten & McKnight 1989; Hansson et al 1999), offering support for PKA-I being more responsive to cAMP. It cannot be ruled out, however, that the PKA might be affected in the post-mortem state leading to reduced responsiveness to cAMP in comparison to living tissue (ie. BLCLs). It is noteworthy, however, that in a recent study reporting on PKA activity in post-mortem brain, PKA activity did not correlate with post-mortem delay suggesting that this factor did not influence the findings of the study (Fields et al 1999).

The mean Hill slope for cAMP-stimulated PKA activity in BLCLs from all subjects was 1.45 ± 0.29, reflecting the positive cooperativity that has been well described for the
sequential binding of cAMP to the two sites on the R-subunits of PKA. The estimated Hill
slopes obtained in this study agree well with the reported range of values for cAMP-
binding to PKA: 1.4-1.6 (Bubis & Taylor 1987).

IV.1.4 Inhibition of Phosphodiesterase Activity

The non-specific cAMP PDE inhibitor, IBMX (Beavo et al 1970; Montague &
Cook 1971) has often been used in assays of PKA activity to prevent the degradation of
cAMP during the incubation period for stimulation. IBMX is a competitive inhibitor that
shares considerable structural homology with cAMP (Tomes et al 1993). Inclusion of
IBMX (500 µM) in the BLCL activity assay in this study did not significantly affect basal
or maximally-stimulated PKA activity, however. In fact, there was a trend towards lower
estimates of maximally-stimulated PKA activity in the presence of IBMX compared with
the PKA activity measured in the absence of the PDE inhibitor, contrary to what might be
expected. These findings can be compared to the measurement of PKA activity in post-
mortem brain where inclusion of IBMX (500 µM) in the reaction mixture also did not
result in higher estimated values for PKA activity (Fields et al 1999).

One possible explanation for the ineffectiveness of IBMX in the assay is that the
added cAMP in the maximally-stimulated condition is already in excess providing
saturating cAMP concentrations for both PKA and PDEs. This might be expected to render
the action of the PDEs inconsequential. This explanation, however, is only applicable to
cAMP-stimulated conditions and cannot explain the lack of effect of IBMX in the basal
condition. Another possibility, applicable to both the cAMP-stimulated and basal
conditions is that some component of the tissue preparation might degrade PDEs, thereby reducing their effects.

One further possibility for the ineffectiveness of IBMX in this preparation is suggested by evidence that IBMX may have some untoward effects on PKA activity. In cAMP-stimulated conditions, IBMX has been shown to strongly inhibit the binding of cAMP to PKA through its role as a cAMP analogue (Tomes et al 1993). In basal conditions, IBMX was shown to slightly increase basal PKA activity. These findings highlight a possible role for IBMX as a partial agonist for PKA activity and suggest that the addition of IBMX to assays of PKA activity might result in inaccurate estimates of PKA activity (Tomes et al 1993). This possible role for IBMX as a partial agonist could explain the trend for slightly lower maximally-stimulated PKA activity in the presence as compared to the absence of IBMX. That is, the PDE inhibitor might be competing with the added cAMP resulting in less apparent activation of PKA and thus lower PKA activity. Together the results indicate that the addition of IBMX to the BLCL PKA activity assay does not increase measurable PKA activity and might actually interfere with the activation of PKA. Given these findings, IBMX was not included in subsequent BLCL PKA activity assays.

IV.1.5 Concentration-Response Relationship of PKA Activity for Kemptide

The relationship of PKA activity with respect to Kemptide concentration revealed a single site binding hyperbolic relationship of PKA activity for a concentration range of 2 μM-1 mM. The estimated mean Km for Kemptide was 11.1 ± 1.7 μM (n=3), well within the observed range of Km values reported for other cell types. For example, estimates for
the Km in bovine heart was 4.7 μM (Whitehouse et al 1983), whereas it was 16 μM in bovine skeletal muscle (Kemp et al 1977) and 20 μM in human platelets (Hatmi et al 1996). To ensure that saturating concentrations of Kemptide would be used in assays of BLCL PKA activity, a concentration of Kemptide was chosen that was approximately 10 times the observed Km (100 μM). At this concentration of Kemptide maximum substrate utilization was never greater than 15% in the assay conditions employed, further supporting the choice of concentration of 100 μM.

IV.1.6 Linearity of PKA Activity with Respect to Protein

Both the basal and maximally-stimulated PKA activity in cytosolic fractions increased in a linear fashion with respect to the amount of protein in the range of 2-10 μg. While first-order polynomial equations gave excellent fits to the data for both parameters, the relationship in the maximally-stimulated condition was better fit by a second-order polynomial equation due to the slight curvilinear component to the data. Examination of the curvilinear component showed that at amounts of protein exceeding 8 μg, the relationship between PKA activity and protein began to deviate from linearity, although subtly. This suggested that amounts of protein greater than 8 μg were approaching saturation. Accordingly, in subsequent assays the amount of protein used in the reagent mixture was targeted to the mid-point of the linear range of activity with respect to protein (4-5 μg). The protein-dependent increases in PKA activity, however, do indicate that for the amount of PKA present in the tissue fraction the other reagents are of saturating concentrations.
IV.1.7 Linearity of PKA Activity with Respect to Time

PKA activity showed a linear relationship with respect to time for both the basal and maximally-stimulated conditions from 1-20 min. The mid-point of this range, 10 min, was chosen as the incubation time for subsequent experiments. An incubation time of 10 min has been widely used by other investigators (Roskoski 1983; Glass et al 1989; Rohlff et al 1993). The time-dependent linear increases in PKA activity also support that both Mg-ATP and Kemptide were at saturating concentrations.

IV.2 Characterization of PKA Activity in BLCLs

IV.2.1 Comparison of BLCL PKA Activity in BP-I High Ca$^{2+}$, BP-I Normal Ca$^{2+}$ and Healthy subjects

Basal PKA activity, maximally-stimulated PKA activity and EC50 for cAMP activation of PKA were determined in BLCLs from BP-I High Ca$^{2+}$, BP-I Normal Ca$^{2+}$ and healthy subjects to evaluate if PKA activity is increased in cells from BD patients in comparison to healthy subjects, and if these differences are associated with the elevated intracellular [Ca$^{2+}$]$_B$ "endophenotype" found in a significant percentage of BP-I patients. Although maximally-stimulated PKA activity was 9% lower in the BP-I High Ca$^{2+}$ group in comparison to healthy subjects, this difference did not reach statistical significance. Moreover, the slight reduction in activity does not support the hypothesis that PKA activity would be increased as was reported in post-mortem brain (Fields et al 1999) and platelets (Perez et al 1995) from BD patients. There were also no statistically significant differences found in basal PKA activity and EC50 for cAMP activation of PKA activity between BD patients and healthy comparison subjects. Although no statistically significant differences
were found, the possibility of Type II error cannot be ruled out. For this reason, it is still possible that the difference in maximally-stimulated PKA activity between the BP-I High Ca\(^{2+}\) patients and the healthy subjects, although small, might be clinically relevant and merits further testing in a study with a larger sample size.

Collapsing the BP-I High Ca\(^{2+}\) and BP-I Normal Ca\(^{2+}\) patients into a single group also did not reveal any statistically significant differences in the PKA activity measures between BD patients as a whole, and healthy subjects. This indicates that there is no difference in the maximally-stimulated PKA activity in BD patients in general, and that the failure to find statistically significant differences was not attributable to the categorization of the BP-I patients into subgroups based on a high and normal intracellular [Ca\(^{2+}\)]\(_{\text{B}}\) endophenotype.

The lack of differences in BLCLs from BD patients compared with healthy subjects contrasts with the findings of Fields et al (1999) in which changes were found in PKA activity measures from post-mortem brain of BD patients compared with matched controls. Specifically, the latter investigators found higher basal and maximally-stimulated PKA activity in cytosolic fraction of temporal cortex, and a significantly lower EC50 for cAMP activation of PKA activity in both cytosolic and particulate fractions from temporal cortex of BD patients compared with controls. In the context of the decreased cAMP binding found in post-mortem brain from these same BD patients in comparison with non-neurological non-psychiatric control subjects (Rahman et al 1997), these alterations in PKA activity in post-mortem brain could indicate less R-subunits and thus more free C-subunits available for activity (Fields et al 1999). This hypothesis is supported by the reported association of a reduced amount of R-subunits with long-term sensitization in Aplysia
suggesting that a reduced R- to C-subunit ratio may lead to hyperfunctional PKA activity. That is, subsaturating concentrations of cAMP can more effectively activate the enzyme (Greenberg et al 1987). Similar to the findings of increased maximally-stimulated PKA activity in post-mortem brain, increased cAMP-stimulated phosphorylation of an endogenous substrate has been shown in platelets from BD patients (Perez et al 1995) supporting the hypothesis of altered PKA functionality in BD.

One possible explanation for the contrasting results found with the BLCL model in comparison to post-mortem brain and platelets might be related to differences in PKA composition and activity. The isoforms of PKA are differentially expressed in a tissue- and subcellular-specific manner, and also show unique roles in cellular function as well as properties of regulation (reviewed in Francis & Corbin 1999; Hansson et al 1999). Important differences in subunit expression exist between neural and peripheral tissues; β-subunits are expressed at higher levels in the former tissue whereas α-subunits are expressed to a greater extent in the latter (Uhler et al 1986; Scott et al 1987; Clegg et al 1988; Doskeland et al 1993; Guthrie et al 1997; Brandon et al 1998). It is possible, therefore, that alterations in PKA activity might occur in a manner unique to BLCLs, or that the putative causes of abnormal PKA activity in post-mortem brain and platelets from BD patients might interact differently with the specific PKA isoforms in BLCLs from BD patients. For example, the increased maximally-stimulated PKA activity in post-mortem brain (Fields et al 1999) might be an adaptive response to up-stream disturbances related to increased Gsα subunit levels and activity (Young et al 1993). The specific changes that PKA may undergo are known to be profoundly influenced by the type of tissue (Ratoosh et al 1987; Oyen et al 1988; Houge et al 1990; Spaulding 1993; Hansson et al 1999).
Sustained cAMP elevations in primary rat hepatocyte cultures resulted in a down-regulation of C-subunit levels and increased levels of RIα and RIIα mRNA (Houge et al 1990), while in ovarian granulosa cells exposure to cAMP resulted in increased levels of RIIβ mRNA and protein (Ratoosh et al 1987). Therefore, it is possible that in BLCLs, in contrast to post-mortem brain, PKA might be able to compensate for putative up-stream disturbances in signal transduction through changes in gene transcription or subunit mRNA stability in response to abnormal signaling through cAMP- or Ca²⁺-mediated pathways. Such compensation might result in the "normal" PKA function found in BLCLs of BD patients.

Alternatively, the lack of statistically significant differences in BLCL PKA activity between BD patients and healthy subjects might be related to other characteristics of the BLCL model. The model involves the growth of a small number of cells in culture for 13-16 passages. In the cultured environment, the BLCLs are isolated from state factors present in vivo such as hormones and medications which can have profound effects on PKA activity. For example, Li has been shown to increase the basal and cAMP-stimulated phosphorylation of endogenous substrates in the platelets of BD patients (Zanardi et al 1997). In the BLCL model, the small number of progenitor B cells expand in culture at least ten-fold during the 13-16 passages so that more than 90% of the cells at this number of cell divisions are new cells which have grown in the cultured environment. Differences detected in the BLCL model, therefore, would be trait- rather than state-related abnormalities. Indeed, intracellular [Ca²⁺]ₜ (Emamghoreishi et al 1997), increased levels of Gsα subunits (Emamghoreishi 1998), blunted responses to isoproterenol-stimulated cAMP formation and increased basal and NaF-stimulated cAMP production
(Emamghoreishi et al in press), reduced agonist-induced downregulation of decreased β-AR density (Kay et al 1993) and abnormalities in Na+, K+-ATPase activity (Cherry & Swann 1994) have been reported in BLCLs from BD patients (Gα subunit increases were only shown in BP-II patients) in comparison to control subjects. These findings suggest trait-related disturbances in signal transduction in BLCLs of BD patients and support the utility of the BLCL model for the study of the pathophysiology of BD.

In contrast to the BLCL model, PKA activity in post-mortem brain or platelets could readily be influenced by state-related factors that affect the cellular environments, which could confound the interpretation of findings from such studies. Recent evidence supports the occurrence of state-related abnormalities in the cAMP-mediated signal transduction pathway. For example, the levels of C-subunits were elevated in platelets from BD patients in depressed or manic states but not in the euthymic state, in comparison to healthy controls (Perez et al 1999). In addition, in MNLs of BD patients in a manic state the levels of Gα and Gβγ were significantly increased, as was the agonist-stimulated binding capacities of Gpp(NH)p. In MNLs of BD patients in a depressed state the same measures were significantly decreased compared with a control group (Avissar et al 1997a, b). These findings offer support for the influence of state-dependent factors on the cAMP-signal transduction cascade in various tissues in BD.

Taken together, the lack of statistically significant differences in BLCL PKA activity between BD patients and controls, and the evidence supporting state-related abnormalities of signal transduction in BD suggest that putative PKA abnormalities in BD may be state-dependent, as was also concluded by Perez et al (1999). The BLCL model,
therefore, would not allow the elucidation of those differences as the model obviates the influence of state-related factors.

Comparison of BP-I patients with and without lifetime histories of comorbid psychiatric diagnoses did not reveal any statistically significant differences in any of the PKA measures examined. However, stratifying BP-I patients by type of comorbid diagnosis, that is anxiety disorders or substance abuse, revealed that patients with a comorbid diagnosis of an anxiety disorder showed higher maximally-stimulated PKA activity than those with a comorbid diagnosis of substance abuse.

Increasing evidence from phenomenological, neurobiochemical and neuropharmacological studies support the notion that BD is a heterogeneous group of illnesses (Goodwin & Jamison 1990). BD exhibits heterogeneity in clinical features such as different peak ages of onset, course modifiers (eg. rapid cycling), presence or absence of psychotic features, mixed or dysphoric states, responsiveness to medications and comorbid psychiatric diagnoses (Goodwin & Jamison 1990; DePaulo Jr & McMahon 1996). Heterogeneity of the disorder has also been suggested based on biochemical measures such as intracellular $[Ca^{2+}]_B$ (Emamghoreishi et al 1997), and IMPase activity (Shamir et al 1998). It has been suggested that BD patients categorized by specific diagnostic characteristics may identify separate subtypes of BD with specific genetic vulnerabilities or pathophysiological mechanisms (Gershon 1990; Potash & DePaulo 2000). For example, the activity of IMPase was significantly lower in BLCLs from BD patients in comparison to healthy subjects (Shamir et al 1998). Stratifying the patients by Li responsiveness revealed that those patients who were Li responders showed significantly lower BLCL IMPase activity than those patients who were poor Li responders suggesting heterogeneity
within BD (Shamir et al 1998). That is, low IMPase activity and Li responsiveness may distinguish a specific phenotype within BD. Similarly, it has been suggested that comorbidity with substance abuse distinguishes a subgroup of BD patients who differ genetically and/or in pathophysiology from BD patients without a history of substance abuse (Potash & DePaulo 2000). In support of this notion, intracellular $[\text{Ca}^{2+}]_i$ levels in BLCLs from BP-I patients with comorbid alcohol abuse were lower than those in BP-I patients without comorbid substance abuse but similar to the levels found in healthy subjects (Emamghoreishi 1998). The findings in the current study, although preliminary, add further support to this notion, suggesting that BD patients with a comorbid diagnosis of an anxiety disorder might represent a subtype of BD patients different from those with a comorbid diagnosis of substance abuse.

Interestingly, for 14 of the 17 patients in the study with comorbid psychiatric diagnoses of substance abuse, the primary or sole abused substance was alcohol. Thus, the difference in PKA activity that was found might represent a vulnerability factor specific to BD patients who abuse alcohol that may be relevant to the pathophysiology of the disorder in this BD subgroup. Alternatively, the lower maximally-stimulated PKA activity in this group compared with BD patients with an anxiety disorder might be explained by the effects of alcohol on PKA and the cAMP-mediated signal transduction system (reviewed in Diamond & Gordon 1997).

Clinical studies have found reduced signaling in the cAMP-mediated signal transduction pathway in various tissues from abstinent alcoholic patients. Lymphocyte membranes from short-term abstinent alcoholics showed increased levels of both protein and mRNA of Gαi, increased levels of Gαs mRNA, as well as decreased levels of both
basal and prostaglandin E1- and GTPγS-stimulated AC activity in comparison to healthy controls (Waltman et al. 1993). Lower receptor- and Gs-stimulated AC activity was found in platelets of alcoholic subjects abstinent from alcohol for 12-48 months in comparison to healthy subjects (Tabakoff et al. 1988). In addition, lower cholera-toxin-catalyzed [32P]ADP-ribosylation of Gs was found in the platelets of abstinent alcoholics in comparison to healthy subjects (Manji et al. 1992). Together, the findings suggest long-lasting, possibly epigenetic effects of alcohol on the cAMP system. Alternatively, the findings could suggest that abnormalities in the cAMP signal transduction system may represent underlying genetic differences in alcoholic patients (DePaulo & McMahon 1996; Potash & DePaulo 2000).

Possible mechanisms for the effects of alcohol on PKA are suggested by studies exploring the effects of chronic ethanol exposure on cellular processes. While still controversial, there is accumulating evidence to suggest that chronic ethanol exposure does indeed cause reductions in activity in the cAMP-mediated signal transduction pathway (Diamond & Gordon 1997). For example, in S49 cells, chronic exposure to ethanol resulted in decreased PKA activity and a loss of inhibition of adenosine uptake that was reversed through the activation of PKA (Coe et al. 1996). In NG108-15 cells, ethanol incubation (48 h) caused increased translocation of Ca-subunits to the nucleus that persisted until ethanol exposure was discontinued, as well as reductions in RI-subunit levels (Dohrman et al. 1996). Together the findings suggest that chronic alcohol abuse may cause hypofunctionality within the cAMP-mediated signal transduction pathway. The findings in this study of lower PKA activity in BD patients with a comorbid diagnosis of substance abuse (notably alcohol), in comparison to patients with anxiety disorders are
consonant with the latter notion, particularly if these induced effects are sustained in the absence of ethanol as suggested by the studies of abstinent alcoholics (Tabakoff et al 1988; Manji et al 1992; Waltman et al 1993).

The influences of age of the subject, intracellular $[\text{Ca}^{2+}]_B$, and duration of freezer storage on the PKA activity measures were, for the most part, ruled out by the absence of significant correlations between these factors and the PKA-dependent measures. However, there was a modest correlation between duration of freezer storage and maximally-stimulated PKA activity. This correlation was lost, however, with the removal of two subjects defined as outliers as their cells had been stored at -70°C for 36 and 49 months respectively, greater than two SDs beyond the mean length of freezing time for the sample population as a whole. This suggests that the duration of freezer storage did not have a significant effect on PKA activity for time periods ≤ 20 months. Although longer storage times may be associated with lower maximally-stimulated activities, it is logistically impossible to grow fresh cells for such extended periods of time to enable an accurate comparison between fresh and frozen cells.

A modest but statistically significant correlation between intracellular $[\text{Ca}^{2+}]_B$ and the EC50 for cAMP activation of PKA was also strongly influenced by the results from two BP-I High $\text{Ca}^{2+}$ patients defined as outliers (EC50 measures which were also more than two SDs below the mean of controls). The removal of these outliers resulted in the loss of significance for the correlation of intracellular $[\text{Ca}^{2+}]_B$ and EC50. Thus, this correlation was also of questionable significance, especially in the absence of statistically significant differences in EC50 values between the BP-I High $\text{Ca}^{2+}$, BP-I Normal $\text{Ca}^{2+}$ patients and
controls. However, the basis for the low EC50s of the two BP-I High Ca\(^{2+}\) patients (14.76 and 14.57 nM) (35% of the healthy subjects) may be of interest as they could reflect a smaller subgroup of BD patients and suggest the importance of increasing the number of subjects to determine the prevalence of the EC50 and intracellular [Ca\(^{2+}\)]\(_B\) relationship.

IV.2.2 Comparison of BLCL Activity in MDD, BP-II and Healthy Subjects

Similar to the findings for the BP-I patients, there were no statistically significant differences found in any of the PKA measures obtained in BLCLs from MDD and BP-II patients compared with healthy subjects. However, mean maximally-stimulated PKA activity was 10% lower in BLCLs from BP-II patients compared with healthy subjects. The percent difference is similar to that found between BP-I High Ca\(^{2+}\) patients and healthy subjects. The lack of statistical significance might similarly be attributable to a Type II error.

The lack of statistically significant differences in PKA activity between the MDD patients and healthy subjects contrasts with the findings of studies performed on cultured fibroblasts from MDD patients (Manier et al 1996; Shelton et al 1996). In those studies, maximally-stimulated PKA activity was lower in fibroblasts from MDD patients compared with control subjects, without a difference in EC50 for cAMP activation of PKA (Manier et al 1996; Shelton et al 1996). These contrasting findings may reflect tissue-specific differences in PKA responses as discussed above in regards to post-mortem brain and BLCLs.

To my knowledge, PKA activity has never before been examined in tissues or blood cells of BP-II patients and thus there are no findings with which to compare the results of
this study. However, increased levels of Gsα have been shown in the BLCLs of BP-II patients compared to healthy controls (Emamghoreishi 1998) and suggest that altered cAMP-mediated signal transduction may occur in BLCLs of these patients. Similarly, increased levels of Gsα have been shown in the platelets of BP-II patients compared with healthy controls (Mitchell et al 1997). The lack of changes in PKA activity in BLCLs from BP-II patients found in this study could be explained as above. That is, compensations may occur in PKA activity in the BLCLs from BP-II patients to adapt to putative upstream disturbances in cAMP signaling through modifications of gene transcription or protein stability as considered earlier. Alternatively, the lack of increased levels of intracellular 
$[Ca^{2+}]_b$ in BP-II as compared with BP-I patients (Emamghoreishi et al 1997) suggests that different underlying pathophysiological disturbances may occur in BP-II disorder. Therefore, BP-II patients might not be expected to show disturbances in PKA function of the same nature as BP-I patients thereby explaining the negative findings in this study.

Including the state of illness of the MDD or BP-II patients in the analyses in this study revealed a significant interaction between sex and state. Contrasts of sex within state showed that maximally-stimulated PKA activity in BLCLs from male patients in a euthymic state was significantly lower than that in male patients who were ill at the time of study (depressed or manic). Additionally, it was found that maximally-stimulated PKA activity of female patients in an illness state was significantly lower than for male patients in an illness state. Of note, if the state of the BP-I patients was included as a factor in the statistical analyses of the data there was a trend for higher maximally-stimulated PKA activity in BLCLs from female patients as compared with male patients.
These findings are difficult to reconcile with the BLCL model and suggest that the observed statistically significant differences may represent sampling artifacts. Alternatively, it is conceivable that the B lymphocytes from patients in illness or euthymic states may be differentially affected by the EBV transformation. In turn, this differential effect might be influenced by the sex of the patient. A second possibility is that the state of the patients might be reflective of the severity of the disorder. That is, the patients that were ill at the time of the study might present with a more severe form of the disorder and are thus more likely to be in a sustained state of illness compared with those patients who were euthymic at the time of the study. It is important to mention that possible sex effects on biologically dependent variables in BD have been suggested by previous studies examining intracellular $[Ca^{2+}]_\text{b}$ in T lymphocytes (Emamghoreishi et al 1997) and the levels of IMPase2 mRNA expression in BLCLs (Yoon et al submitted). It was suggested, however, that the ostensible sex differences might in fact be related to extraneous factors such as responsiveness to medication (Yoon et al submitted) or confounding effects of medication (Emamghoreishi et al 1997). As there were no sex effects found in all of the other analyses of PKA activity measures in the study the relevance of the sex and state interactions is uncertain. As the small numbers of subjects in the analyses could result in sampling artifacts an expanded study with a larger number of subjects is needed to clarify the relevance of the findings unequivocally.

There were no statistically significant differences for any of the PKA activity measures between the MDD and BP-II patients stratified by a lifetime history of a psychiatric comorbid diagnosis, nor for patients stratified by the type of comorbid psychiatric diagnosis (anxiety disorder or substance abuse). Thus, these diagnostic
characteristics do not appear to be associated with differences in BLCL PKA activity from MDD and BP-II patients. Furthermore, age, intracellular [Ca\textsuperscript{2+}]\textsubscript{b} and duration of freezer storage did not correlate with basal PKA activity, maximally-stimulated PKA activity or the EC50 for cAMP activation of PKA. This suggests that these factors are not likely to have confounded the results of the analyses of PKA activity in MDD and BP-II patients.

IV.2.3 Comparison of BLCL PKA Activity in BP-I High Ca\textsuperscript{2+} and BP-I Normal Ca\textsuperscript{2+} Patients with MDD and BP-II Patients

As described in the results an adjustment factor was applied to the data from MDD and BP-II patients to correct for the interbatch assay effect. The comparison of the four patient groups did not reveal any statistically significant differences in basal or maximally-stimulated PKA activity in BLCLs of the patient groups. The findings do not agree with those obtained in studies of post-mortem brain and fibroblasts, which suggest that PKA activity might be increased in BD (Fields et al 1999) and reduced in MDD patients (Manier et al 1996; Shelton et al 1996). Furthermore, the findings of increased levels of Gs\textalpha\ in the MNLs from depressed BD patients compared with healthy controls (Young et al 1994), and decreased levels in MNLs from MDD patients (Avissar et al 1997a) also suggest that hyperfunctional cAMP signaling occurs in BD patients and hypofunctional signaling in MDD patients. As discussed, however, abnormalities found in post-mortem brain and MNLs could reflect state-related changes that are not manifest in BLCLs. Alternatively, differences in PKA response unique to BLCLs might compensate for any possible upstream abnormalities differentially in BD as compared with MDD patients, resisting potential changes in net PKA activity.
The EC50 for cAMP activation of PKA was higher in BLCLs from BP-II patients than BP-I High Ca²⁺ patients, although the magnitude of the difference was relatively small (29%). The higher EC50 was not associated with a difference in Hill slopes for the concentration-response relationships of PKA activity for cAMP between the two patient groups and thus, was not indicative of reduced positive cooperativity. The difference in EC50, therefore, reflects a small shift to the right in the concentration-response relationship for cAMP-stimulated PKA activity in BLCLs from BP-II patients but its biological relevance is uncertain given the small magnitude. Further examination of the EC50 in experimental conditions that more closely resemble in vivo environments might allow for the elucidation of the relevance of the finding.

Unlike the separate analysis of the MDD and BP-II patients, there were no effects of state of illness of the patients on PKA activities in BLCLs from all patients analyzed as a whole (BP-I, BP-II and MDD). Furthermore, analyses of the patients as a group did confirm a general effect of type of past history of comorbid psychiatric diagnosis on maximally-stimulated PKA activities in mood disorder patients. As the analysis of just the MDD and BP-II patients showed no effect of comorbidity type this difference was attributed to the BP-I group, as discussed earlier, and not BP-II and MDD patients. Therefore, the finding is likely diagnosis specific.
IV.3 Age of Onset

Increasing evidence suggests that age of onset of BD might be an important demographic characteristic that is relevant to the pathophysiology of the disorder. Age of onset of illness correlated significantly with maximally-stimulated PKA activity for BD patients as a whole (BP-I High Ca^{2+}, BP-I Normal Ca^{2+}, BP-II) and for BP-I patients (BP-I High Ca^{2+}, BP-I Normal Ca^{2+}) examined separately. Studies of age of onset in BD indicate a main peak age of onset in the 15-19 year age range (early onset) followed by a second peak for age of onset in the 20-24 year age range (adult onset) (Goodwin & Jamison 1990). The patients were consequently subdivided into two subgroups: early onset (\leq 19 years) and adult onset (\geq 20 years). Further comparisons with age of onset as a categorical factor (ie. early versus adult onset) confirmed significantly higher maximally-stimulated PKA activity (18\%) in early onset BD patients.

Studies examining possible differences in clinical outcome and pathophysiology between early onset and adult onset BD patients have yet to fully establish the relevance of the subgrouping of the patients by this variable (Goodwin & Jamison 1990; McMahon & Depaulo 1996). Several studies have noted an association between psychotic features and earlier ages of onset (Angst 1986; McMahon & Depaulo 1996). Indeed, a recent study has shown that early onset (\leq 18 years) BD patients with psychotic symptoms exhibit increased frequency of the apolipoprotein E4 allele compared to BD patients with early onset of illness without psychotic symptoms, BD patients with later age of onset of illness, and controls (Bellivier et al 1997) adding to evidence suggesting the relevance of subgrouping BD patients by age of onset.
Age of onset studies have also provided evidence for anticipation, the observation that affected children tend to have lower ages of onset and greater illness severity than their affected parents (McMahon & Depaulo 1996; Ohara et al 1998). A genetic basis for this phenomenon could be triplet repeat expansion (O’Donovan & Owen 1996), that is the increase in size of a repeated sequence of trinucleotides in successive generations of family members resulting in earlier expression of the disease and possibly greater severity (McMahon & Depaulo 1996; O’Donovan et al 1996; Mendlewicz et al 1997). Triplet repeat expansion has been observed in other diseases including myotonic dystrophy (Fu et al 1992; Harley et al 1992), Huntington’s disease (Huntington’s Disease Collaborative Research Group, 1993) and Fragile X Mental Retardation (Verkerk et al 1991).

Interestingly, several studies using the repeat expansion detection (RED) method have shown associations between longer CAG/CTG repeats and BD (O’Donovan et al 1996; Craddock et al 1997) although the observations are controversial as others have failed to show such an association (Guy et al 1997; Li et al 1998). It remains to be shown, however, that early onset BD patients are associated with longer CAG/CTG repeats than adult onset BD patients (O’Donovan et al 1996). Indeed, differences in maximally-stimulated PKA activity might be an important marker of underlying differences in trinucleotide repeat lengths between the two subgroups.

Taken together, it is evident that a study specifically designed to address the relationship between age of onset and PKA activity must be conducted to allow more precise conclusions to be made about the relevance of subtyping BD patients by age of onset. In addition, future studies examining the association of trinucleotide repeat
expansion with age of onset may serve to further establish the relevance of stratification by the onset factor.

IV.4 Limitations of the BLCL Model

The use of the BLCL model to study putative abnormalities in PKA activity in BD might be a possible limitation of this study. Given that the cells are of non-neuronal lineage, they may not express abnormalities in neuronal signal transduction, as occur in BD brain. However, the demonstration of signaling abnormalities in peripheral cells from BD patients (Dubovsky et al 1992; Young et al 1994; Perez et al 1995; Perez et al 1999) argues that some component that affects intracellular signaling is also expressed in peripheral cells.

An additional aspect of the model that may have affected the results of the study is the transformation of the lymphocytes with EBV. Immortalizing the cell lines might in its own right alter PKA, which is involved in cell cycle regulation (Francis & Corbin 1999), thereby masking any abnormalities in PKA activity in BD. However, the higher intracellular [Ca²⁺]ᵢ found in MNLs, platelets (Dubovsky et al 1992) and BLCLs from BD patients (Emamghoreishi et al 1997) does support the utility of the model for elucidating Ca²⁺-related disturbances in BD. The lack of overall statistically significant findings in PKA activity in BLCLs from BD patients compared with controls contrasts with findings in postmortem brain (Fields et al 1999) and platelets (Perez et al 1995; 1999). This may reflect the inadequacy of the BLCL model for elucidating disturbances in downstream cAMP signaling. Therefore, it is not possible to ascertain unequivocally whether the lack of overall statistically significant differences in this study are because previously reported abnormalities in PKA activity are state-related, or alternatively, because of the model itself.
IV.5 Conclusions

This study has successfully adapted a procedure to assay PKA activity in BLCLs based on the use of a PKA-specific inhibitor to define that activity which is PKA specific. Under the conditions described, PKA activity in BLCLs from healthy subjects exhibited a mean $K_m$ for Kemptide of $11.1 \pm 1.7 \mu M$. Maximal stimulation of PKA by cAMP occurred at 500 nM, with an EC50 of $30.48 \pm 3.89$ nM and mean basal and maximally stimulated PKA activities of $0.16 \pm 0.09$ pmol/min/µg protein and $3.28 \pm 1.14$ pmol/min/µg protein, respectively, for healthy subjects. Seventy percent of PKA activity was found in the cytosolic fraction of BLCLs fractionated at 48,000 g, whereas only 30% was associated with the particulate. This cellular distribution, along with the kinetic parameters obtained, suggests PKA in these cell lines is remarkably similar to that reported for other cell lines of peripheral tissue origin, in contrast to post-mortem brain. To my knowledge, this is the first study to systematically characterize PKA in BLCLs.

The study is also the first time PKA activity has been determined in BLCLs from BD and MDD patients, and healthy subjects.

Comparison of PKA activity in the BLCLs from BD patients phenotyped on primary DSM-IV criteria of BP-I disorder or BP-I disorder with elevated BLCL intracellular $[Ca^{2+}]_i$ did not reveal differences from the activity obtained in BLCLs from healthy subjects as hypothesized. Similarly, no significant differences were observed in PKA activity between the illness phenotypes of BP-II or MDD and healthy subjects. Small differences in EC50 for cAMP activation of PKA were found between BP-II and BP-I High $Ca^{2+}$ patients, although the relevance of this finding is unclear. Given that the BLCL model
would be expected to reflect only fixed disease-induced or trait-related alterations, putative PKA abnormalities in BD as suggested by alterations in postmortem and platelet studies might be in part state-related disturbances to which the BLCL model would be insensitive. Alternatively, the BLCL model might in some way be masking putative PKA abnormalities in BD which may in fact be trait-related

While the latter phenotypes were not associated with differences in BLCL PKA activity, correlates were identified between the activities within the BD patient sample and two important phenomenological variables, that of age of onset of illness and lifetime history of comorbid anxiety versus substance abuse disorders. This suggests that the relative capacity of PKA activity may segregate differentially with these two putative “endophenotypic” characteristics.

Finally, data was also obtained suggesting possible interactive effects of sex on PKA activity in BLCLs from both BD and MDD patients. As this difference was only evidenced as an interaction with state of illness, however, it remains to be clarified whether this represents a true sex difference or is related more directly to other factors such as illness severity and medication responsiveness, which were uncontrolled extraneous factors in this preliminary investigation.

IV.6 Future Directions

The finding that BLCL PKA activity is similar to that reported for other peripheral tissues, but different from that measured in post-mortem brain, would be complemented by studies exploring the precise isozyme composition of PKA in BLCLs. This knowledge would allow more definitive conclusions to be made about the nature and mode of
regulation of PKA, and its activity in BLCLs. The information could also improve the utility of the BLCL model for the study of PKA activity in a disease relevant condition and to evaluate the effect of various therapeutic agents on this protein in a model with direct relevance to humans. Furthermore, characterization of the isozyme composition of PKA in these cell lines would assist in elucidating whether adaptive changes in the abundance/composition of PKA subunits occur in BLCLs that offset potential alterations in activity and whether these are associated with illness phenotypes.

Expansion of subject number would clearly enhance the power of the study design to detect statistically significant differences hinted at by the small differences in maximally-stimulated PKA activity between BD patients and healthy subjects, and in EC50 for cAMP activation of PKA between BP-I and BP-II patients. A larger sample size would also allow more rigorous testing of the relevance of possible state and sex effects suggested by this study.

To further test if putative alterations in PKA activity are state- and not trait-related, a future study could be carried out in which BLCLs are cultured in the presence or absence of various factors that would stress the cells in a manner representative of in vivo state-related factors. For example, BLCLs could be cultured in the presence of cortisol, which is elevated, at least during active states of illness, in BD patients (Cassidy et al 1998; Vieta et al 1999). Stressing the cell lines of the BD patients could serve to uncover important underlying differences in PKA activity between the patients and healthy subjects that may only be manifested in certain states. The cells could also be cultured in the presence of Li which has been reported to exert differential effects on PKA activity in tissue from BD patients compared with healthy subjects (Zanardi et al 1997). This would allow for the
exploration of the molecular actions of Li (and other mood stabilizers) in a cellular model with potentially direct disease relevance.

The heterogeneity amongst BD patients suggested by the preliminary findings of this study highlights the importance of conducting further studies specifically designed to address the association of PKA activity with BD patient groups subtyped by putative phenotypic characteristics such as lifetime history of comorbid diagnoses and age of onset (and indeed other clinical variables such as response to therapies). Such studies would help to establish more conclusively the pathophysiological relevance and utility of subtyping patients based on biochemical abnormalities such as PKA activity. In addition they would clarify whether relative PKA activity levels represent vulnerability/protective factors modifying the severity and course of these disorders.
CHAPTER V

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