University of Alberta

The Modulation of Cyclic Nucleotides in Rat Pinealocytes by a Tyrosine Kinase Inhibitor, Tyrphostin B42

by 

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Edmonton, Alberta

Fall 1997

Abstract

In this study, the effects of a substrate-site acting tyrosine kinase inhibitor, tyrphostin, on the cyclic nucleotide accumulation in dispersed male rat pinealocytes were examined. Treatment with 300 μM tyrphostin B42 increased both the cAMP and cGMP accumulation above basal levels. The extent of tyrphostin B42-induced cyclic nucleotide accumulation increase was more substantial for cGMP than cAMP, suggesting tonic regulation of these nucleotides is different. Activation of the adrenergic linked cyclic nucleotide pathway using isoproterenol (1 μM), norepinephrine (10 μM) or forskolin (10 μM) elevated cAMP accumulation, which was further elevated in the presence of tyrphostin B42. The use of tyrphostin A1, a less potent tyrosine kinase inhibitor, demonstrated that the observed effects of tyrphostin B42 are likely associated with inhibition of tyrosine phosphorylation(s). The use of isobutylmethylxanthine (IBMX) (1 μM) to inhibit the phosphodiesterase activities revealed that this is one probable site of action of tyrphostin B42, and further, that tyrphostin B42 has a second site of action that results in a decreased cyclic nucleotide accumulation in adrenergically-stimulated and IBMX-treated pinealocytes. This study augments the growing data which demonstrate the involvement of tyrosine kinase activity in G-protein coupled signal transduction pathways.

Key Words: Tyrphostin - Tyrosine kinase activity - Cyclic AMP - Cyclic GMP - Pineal gland - Male rat

Acknowledgement

I would like to acknowledge the Faculty of Medicine 75th Annual Research Scholarship, which covered my initial three-month stipend, and the Heritage Medical Research Studentship, which covered the remainder of my graduate studies stipend as well as an annual laboratory stipend. Accordingly, I would like to thank the Medical Research Council, which paid for the various research expenses, and Dr. A.K. Ho for sharing his expertise regarding my project. I would like to thank my entire family, who have continually supported me in my educational endeavours. Special thanks to Brenda, for helping to motivate me in the final stages of this thesis. I dedicate this research to my father, James Rogers Murdoch, B.Sc., who passed away during my graduate studies, and my son, Xavier James Mitchell Murdoch, who was born during my Masters program.

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List of Symbols, Nomenclature and Abbreviations

α:	Alpha
ADP:	Adenosine diphosphate
ATP:	Adenosine triphosphate
β :	Beta
cAMP:	Adenosine 3'5'-monophosphate
cGMP:	Guanosine 3'5'-monophosphate
DMEM:	Dulbecco's modified eagle medium
DMSO:	Dimethyl sulfoxide
FCS:	Fetal calf serum
fmole:	Femtomoles
FSK:	Forskolin
g:	Gram
G-protein:	Guanine-nucleotide binding proteins (hetero trimer)
G_{α} :	α subunit of G-protein
$G_{\beta\gamma}$:	β and γ subunit of G-protein (dimer)
GDP:	Guanosine diphosphate
GTP:	Guanosine triphosphate
hr:	Hour
IBMX:	3-Isobutyl-1-methylxanthine
Iso:	Isoproterenol
M:	Molar

µG:	Microgram
µM:	Micromolar
min:	Minute
mL:	Milliliter
mM:	Millimolar
NaAc:	Sodium acetate buffer
NE:	Norepinephrine
NSB:	Non-specific binding
PBS:	Phosphate buffered saline
PDE:	Phosphodiesterase
pmole:	Picomoles
Pz:	Prazosin
RIA:	Radioimmunoassay
SAR:	Sheep anti-rabbit antibody
SCN:	Suprachiasmatic nucleus
SEM:	Standard error of the mean
TRH:	Thyrotropin releasing hormone
Tyr:	Tyrphostins
Tyr 42:	Tyrphostin B42

I. Introduction

I.1. Pineal Gland

Although the pineal gland was described nearly 2,300 years ago by Herophilus, the current understanding of its function remains a speculative subject. However, a modern review realizes that the pineal is a true secretory structure, which contains an extraordinary number of biologically active substances, including norepinephrine (NE), serotonin, dopamine, melatonin, thyrotropin releasing hormone and many others (Laitinen *et al.*, 1995). The pineal gland is embryologically developed from the ependyma lining of the third ventricle and, in the adult rat, lies in the groove between the superior colliculi (Axelrod, 1974). This location makes it accessible and well-recognizable for post-humus surgical excision. In lower vertebrates, like fish, the pineal gland is a true light-sensitive structure, but in higher vertebrates, including mammalian species, the development of a bony cranium has predicated the pineal's light receptor function to become vestigial (Collin, 1971). The mammalian pineal is innervated by a postganglionic sympathetic nerve supply from the superior sympathetic ganglia (Moore, 1973; Lolley *et al.*, 1992). The mammalian pineal is primarily under control of NE message from sympathetic nerves which originate in the superior cervical ganglia (Klein, 1985). The superior cervical ganglion is a component of the following neural pathway: retina - retinohypothalamic projection - suprachiasmatic nuclei - paraventricular nuclei - superior cervical ganglia - nerve conarii - pineal gland (Klein, 1985). This sympathetic nerve inner-

vation, consisting of noradrenergic fibers that end in the interstitial space of the gland or on the pinealocyte plasma membrane, is crucial to the regulation of pineal function (see Fig. I.1). The pinealocytes are organized into cords and rest on a basement membrane in relationship to an interstitial space. This is in addition to the fenestration of the pineal gland allows exit and entry of large molecules to and from the interstitial space of the gland. Thus, the pineal gland is well designed for secretory activity (Lolley *et al.*, 1992).

The most well characterized hormone that is synthesized and secreted by the pineal is melatonin. Melatonin is synthesized from tryptophan within pineal parenchymal cells via a stepwise synthetic pathway which includes 5-hydroxytryptamine, serotonin, and N-acetylserotonin intermediates (see Fig.I.2).

Although the mammalian pineal is no longer light receptive, it is known that melatonin release is still influenced by photoperiod. It has been reviewed that the suprachiasmatic nucleus (SCN) receives a direct neural input from the retina, the retinohypothalamic tract, and it is via this neural pathway that external light regulates the superior cervical ganglia and thus influences pineal activity (Reiter, 1991). The synthesis and release of melatonin occurs rhythmically on a 24 hr basis (Klein, 1985). The sympathetic stimulation of pinealocytes via release of the neurotransmitter, NE, stimulates the intracellular accumulation of cyclic AMP (cAMP) via the α-/β-adrenergic-G-protein-adenylyl cyclase-coupled second messenger system. The accumulation of cAMP has been demonstrated to stimulate the transcription,translation and stability of N-acetyltransferase, which is the rate-limiting enzyme

in the synthesis of melatonin (Klein and Weller, 1972; Roseboom and Klein, 1995). It has further been suggested that the diurnal regulation of melatonin synthesis and release is at least partially associated with fluctuations in pinealocyte β-adrenergic receptor density. Specifically, a 24 hr rhythm in rat pineal β-adrenoceptor density was reported such that male rats housed in a 14:10 LD cycle, with light commencing at 0600 hr, a consistent pattern of receptor expression was established. The density of β-adrenoreceptors was low during daytime, with a slight increase prior to lights-off and a peak density at 0200 hr, but decreasing abruptly at 0400 hr (Gonzalez-Brito and Reiter, 1987). This rhythm corresponds well with the observation that during the dark phase (night), melatonin secretion is at its highest (Reiter, 1991). It is imperative to understand that the regulation of cAMP within pinealocytes is a complicated system in which many factors can have an influence, and accordingly many different systems are capable of affecting pinealocyte endocrine activity. Although the most important regulatory stimulus of pineal gland hormone synthesis is light/dark, many different signalling pathways such as phospholipase A_2 and phospholipase C (Sugden *et al.*, 1985), and the Ca^{2+}system (Sugden *et al.*,1986) have been verified as important regulators of pinealocyte cAMP accumulation. Rats under a 14:10 LD schedule and a 50% food restriction showed variations in their serum melatonin levels. While the study showed one week of food restriction did not alter the serum melatonin concentration, long-term (three week) food restriction caused an increase in amplitude of the typical nocturnal melatonin peak and additionally extended the duration of this response (Chik *et al.*, 1987). Therefore,

there is strong evidence that melatonin synthesis and release and its 24 hr rhythm are primarily controlled by environmental LD schedules, but other environmental stimuli such as nutrient availability are also associatively responsible for pinealocyte function. Thus, the pineal gland represents a sympathetically modulated endocrine body which utilizes second messenger systems, especially cyclic nucleotides, to regulate the synthesis of its hormone, melatonin, in a diurnal rhythmic nature. It is of use to review the various aspects of the cAMP signal transduction pathway.

I.2. Cyclic Nucleotides and Signal Transduction

The cyclic nucleotide synthesis pathway can be subdivided into three components: the receptor component, the regulatory component and the catalytic component. The majority of this thesis pertains to the β-adrenergic receptor, G-protein and adenylyl (cAMP) or guanylyl (cGMP) cyclase, respectively. It is well established that utilization of a stepwise activation sequence allows for signal magnification, a key aspect of cell signal transduction processes. For instance, the release of a sympathetic agonist (ligand) binds to its specific receptor(s), the ligand-bound receptor can further activate several regulatory components (G-proteins). The activated G-proteins can each interact with the catalytic peptide (adenylyl cyclase: cAMP, or guanylyl cyclase: cGMP) and the activated enzyme can subsequently convert nucleotide triphosphates to cyclic nucleotide messengers. An elevation in cyclic nucleotide accumulation becomes pertinent since this results in changes in the respective cells' physiological processes. For instance, an elevation in cAMP

results in the stimulation of protein kinase A activity. Activated PKA phosphorylates target proteins which include enzymes, ion channels and transcription factors, resulting in changes in protein function (Cohen, S. 1985). The subsequent sections review each of the signal transduction components.

I.2.1. The β-Adrenergic Receptor

The pineal body with its sympathetic innervation is a good model of a gland which has its secretory activity regulated through adrenergic receptors. It is known that activation of the β-adrenergic receptor causes an increase in cyclic nucleotide accumulation in cultured pinealocytes (O'Dea and Zatz, 1976). The β-adrenergic receptor is a plasma membrane protein containing seven transmembrane domains with its amino terminus on the extracellular side and carboxy terminus on the cytoplasmic side. The β-adrenoceptor is ubiquitously located in all mammalian tissues, and is typically coupled to the activity of adenylyl cyclase (Stiles *et al.*, 1984). One mechanism through which cell sensitivity to catecholamines is regulated is via the adrenergic receptor, either through alteration of the number of receptors in the plasma membrane, or via regulation of the affinity of receptor coupling to other components of the adenylyl cyclase system. The latter involves regulation of receptor interaction or coupling to the adenylyl cyclase via specific interactions with the G-proteins (Stiles *et al.*, 1984). One mechanism for a change in the interaction between receptor and regulatory component is associated with ligand-induced changes in the receptor itself. Of course cell sensitivity to catecholamines is also

modified by post-receptor mechanisms, such as G-protein down regulation. Sibley *et al.* (1985) and Pitcher *et al.* (1992) have demonstrated that, upon ligand binding to the receptor, the receptor is phosphorylated. It was demonstrated that the phosphorylation of the receptor participates in desensitization by contributing to receptor sequestration or reduction in the efficacy of receptor-G-protein interaction (Sibley *et al.*, 1984). Furthermore, an example of desensitization was supported by evidence that mammalian lung β-adrenoceptors were phosphorylated via a cAMP-mediated process (Benovic *et al.*, 1985). In summary, the β-adrenoceptor is a component of the cAMP second messenger pathway that can be regulated. Furthermore, receptor phosphorylation is a modification induced by its ligand that is in part responsible for receptor down-regulation and desensitization. The phosphorylation process affects the receptor's affinity or ability to interact with the regulatory component of the cyclic nucleotide pathway, the G-protein (Pitcher *et al.*, 1992). The G-proteins are the next component of the cyclic nucleotide signal transduction process and, as such, will subsequently be reviewed briefly.

I.2.2. G-Proteins

G-proteins are a family of guanine nucleotide-binding proteins that are membrane bound and serve as a functional regulatory component between receptor and effector subunits of signal transduction pathways. The first suggestion that a G-protein may be involved in transmembrane signalling was initiated by the realization that guanosine triphosphate (GTP) was required for hormonal induction of

adenylyl cyclase (Rodbell *et al.*, 1980). Moreover, an extension of this discovery was that systems were activated upon binding of GTP, whereas GTP hydrolysis was correlated with deactivation (Cassel and Selinger, 1976). An additional observation that resulted in the development of useful pharmacological tools was the capacity of bacterial toxins, cholera toxin and pertussis toxin, to activate specific G-proteins. The G-protein is actually a hetero-trimeric complex which functions as a unit in the transmission of extracellular signals to intracellular second messenger signal. The subunits are classified as α, β and γ, and variations in these subunits are correlated with differences in their respective functions. The hetero-trimeric G-protein responsible for stimulation of adenylyl cyclase is referred to as the G_s protein. The interaction of G_s with an appropriate ligand-bound receptor promotes the binding of GTP the α subunit of G_s. The α subunit then dissociates from the G_s β and γ subunits and interacts with adenylyl cyclase to stimulate its activity (Robishaw *et al.*, 1986). Currently there are about twenty different known mammalian α subunits (Wilkie *et al.*, 1991): four β and six γ subunits (Iniguez-Lluhi *et al.*, 1992). Thus, G-proteins cycle between an inactive GDP-liganded form and an active GTP-liganded form. All isoforms of α subunits are GTPases, but their kinetics for GTP hydrolysis vary from one type of α subunit to another (Carty *et al.*, 1990). Upon GTPase activity, the GTP moiety is degraded into GDP, which allows for recoupling of the G_s alpha subunit with G_s beta-gamma and inactivation of the regulatory component. It is the G_s protein that is activated via cell exposure to cholera toxin, and this procedure was utilized in some of the experiments contained

in this thesis. The cholera toxin covalently links an ADP-ribose to the G-protein, which inactivates the GTPase activity of that G-protein. Since the GTPase can no longer function, the G_s α subunit is not inactivated after it is bound to GTP and thus continues to activate adenylyl cyclase. This site specific action of cholera toxin is a useful tool for investigations into the complex regulation of the cAMP signal transduction pathway. It has also been reported that α subunits of G-proteins may be released from the plasma membrane following activation. Treatment of myoblasts with cholera toxin over periods of greater than 4 to 8 hr resulted in a down-regulation of G_s in the membranes (Milligan *et al.*, 1989).

It has been verified that the rat pineal contains an abundance of Gα subunits, including those specific for G_s, G_i and G_o proteins (Babila *et al.*, 1992), which is indicative of their importance in this tissue. Furthermore, examination of the ontogeny of Gα expression in rat pineals demonstrated neonatal expression; thus, it is transcribed and translated early in life (Babila *et al.*, 1992).

There is currently a diverse group of effectors coupled to G-protein regulators, and Table I.1 summarizes some known G subunit variants and some of their effectors (Conklin and Bourne, 1993).

It has now become evident that many effectors, including adenylyl cyclases, are regulated by both the Gα and Gβγ moieties. G-proteins function as complex regulators of many effectors and, as such, modulate the action of many extracellular messages in terms of their cellular effect. Given the complexity and the ubiquitous expression of G-proteins, it is not surprising that they are potential sites of defects

in signal transduction in some pathophysiological conditions. Bacterial toxins, such as cholera and pertussis, result in covalent modifications of G-proteins that are the basis of the clinical manifestations of such infections. An inherited hormone resistance syndrome, pseudohypoparathyroidism (PHO), was shown to be due to a deficiency in the G_s protein, the regulator of adenylyl cyclase (Spiegel, 1992). Therefore, the physiological role of G-proteins is essential in the maintenance and modulation of effector components of many signal transduction pathways and, as such, is a critical component of cell response and adaptation to extracellular messages. It is one site of action that a drug might influence downstream of cyclic nucleotide accumulations.

The next level in the cAMP signal transduction pathway is the effector, adenylyl cyclase, which will be subsequently reviewed.

I.2.3. Adenylyl Cyclases

Adenylyl cyclases exist as a molecularly diverse group of at least six different mammalian variants. These mammalian adenylyl cyclases plus two non-mammalian isomers are further grouped into five distinct families (Iyengar, 1993). The various adenylyl cyclases do exhibit many common properties, not the least of which includes stimulation by the G_s-protein and similarly activation by exposure to forskolin. The adenylyl cyclases are the primary enzymes that convert ATP to cAMP, and as a group are under the control of a variety of hormones, neurotransmitters and other intrinsic modulators, such as Ca^{2+}/calmodulin. The first adenylyl cyclase

cloned, type 1 adenylyl cyclase, was purified from bovine brain membranes by Pfeuffer *et al.* (1991). It was expressed and shown to exhibit adenylyl cyclase enzymatic activity that was inducible by forskolin and Ca^{2+}/calmodulin (Tang and Gilman, 1992). Adenylyl cyclase isoforms are all approximately 150,000 MW glycosylated proteins. The generalized structure of the six mammalian isoforms show well conserved domains (Gilman, 1987). The generalized structure consists of a short amino terminal region followed by an intensely hydrophobic region containing six transmembrane helices. A cytoplasmic loop separates another hydrophobic region very similar to the prior, and again a cytoplasmic loop which is very similar to the first (Tang and Gilman, 1992). The catalytic domains are located in the cytoplasmic loops and the activity of the adenylyl cyclases is such that perturbations or mutations in either of these loops typically compromises the catalytic activity (Chinkers and Garbers, 1991). The two sets of six transmembrane regions serve to orient the two cytoplasmic loops, but it is also believed to be within the hydrophobic regions that forskolin exerts its effects so as to activate the adenylyl cyclase activity (Gao and Gilman, 1991).

As was eluded to previously, the various subtypes of adenylyl cyclases show variations in their respective regulation by both extracellular and intracellular modulators. The ability to transfect cells with these various types of adenylyl cyclase has allowed for the characterization of their respective regulatory mechanisms. A summary of the various mechanisms of regulation of the mammalian adenylyl cyclase subtypes is reviewed in Table I.2. Of the known adenylyl cyclases, no two

are regulated in the exact same fashion. Furthermore, many of the isoforms exhibit properties of regulation in both stimulatory and inhibitory manners such that it can be imagined that their responsiveness to differing signals would allow fine-tuned regulations of the resultant cAMP accumulations. Thus, the complexity of the regulation of the adenylyl cyclases is consistent with the expected significance of their intrinsic modulation of cAMP and the downstream processes that this second messenger influences. Therefore, the oscillations in intracellular cAMP accumulation can be precisely regulated via specific properties of the adenylyl cyclase. Adenylyl cyclase is therefore another obvious site through which a substance may alter cAMP accumulation.

Insofar as I have reviewed the cAMP second messenger pathway, it is now useful to review the effector component of the cGMP second messenger system, guanylyl cyclase.

I.2.4. Guanylyl Cyclase

The guanylyl cyclase enzymes that catalyze cGMP formation from GTP can also be grouped into multiple families. The guanylyl cyclase enzymes are found associated with the plasma membrane of cells as well as in the cytosol, and these characteristics establish the two families of guanylyl cyclases. The enzymatic activities of the guanylyl cyclases are stimulated via their direct binding of a variety of specific activators. In fact, guanylyl cyclases represent complete signal transducing moieties, with combined signal receptor, signal transducer and enzymatic

effector capacities (Fulle and Garbers, 1994). The membrane associated family of guanylyl cyclases contains at least three isomers, and likely a fourth associated with photoreceptors. These guanylyl cyclases range in size from about 112 kD to about 121 kD, and their extracellular domains act as receptors for the peptide hormones such as atrial natriuretic peptide (ANP; (Tang and Gilman, 1992). There is a short transmembrane domain followed by the intracellular domain which has both a protein kinase-like domain and a catalytic domain (Yuen and Garbers, 1992).

The soluble guanylyl cyclase family consists of two subunits, α and β. Both subunits contain a cyclase catalytic domain but do not possess a tyrosine kinase-like domain nor a transmembrane domain (Garbers, 1992). Coexpression of both α and β subunits is required for catalytic activity, the functional unit being a dimer. The cytosolic guanylyl cyclases are modulated by nitric oxide (NO) accumulations, such that an increase in NO results in activation of the soluble guanylyl cyclases (Mayer, 1994). It is also known that precursors to nitric oxide have the capacity to activate the soluble guanylyl cyclases, and other agents used pharmacologically to stimulate guanylyl cyclases include sodium nitroprusside and nitroglycerin (Yuen and Garbers, 1992). There are at least two different enzymes involved in the synthesis of NO. NO is generated from L-arginine together with L-citrulline by enzymes referred to as NO synthases. One of these requires Ca^{2+}/calmodulin for its activity, and upon cloning from rat brain, the 150 kD protein was found to be homologous to cytochrome P-450 reductase (Bredt *et al.*, 1991). The peptide contains several domains for NADPH, flavin and calmodulin binding, and a potential

cAMP-dependent protein kinase phosphorylation site (Bentley and Beavo, 1992).

There are several interesting aspects of guanylyl cyclase functional design, which include observed desensitization of the membrane-bound guanylyl cyclases, the formation of catalytic domain dimerizations, as well as the potential role of the known kinase-like domains in the membrane-associated guanylyl cyclases. All of these aspects have been studied in recent years (Fulle and Garbers, 1994). Biochemical investigation has demonstrated that sea urchin guanylyl cyclase is a phosphoprotein that is activated by binding of peptides, which also results in dephosphorylation (Bentley *et al.*, 1986). The dephosphorylated membrane-associated guanylyl cyclase is correspondingly desensitized to further stimulation by its peptide ligands (Bentley *et al.*, 1986). Therefore, the guanylyl cyclase receptor/enzymatic activity is regulated, at least in the sea urchin model, by dephosphorylation events. Additionally, serine and threonine residues are known to be phosphorylated in the GC-A type isozyme (Fulle and Garbers, 1994). It is not yet verified whether this phosphorylation may be associated with homologous desensitization processes in this mammalian guanylyl cyclase; however, ANP has been shown to reduce the phosphate content of the GC-A isozyme (Potter and Garbers, 1992). The guanylyl cyclases also contain other potential sites of phosphorylation, including a tyrosine in the kinase-like domain, which may represent other sites that possibly regulate guanylyl cyclase enzyme activity (Garbers, 1992).

The role of the dimerization observed by guanylyl cyclases is undoubtedly associated with their catalytic activity, given the observation that only dimers of the

cytosolic guanylyl cyclases display catalytic activity (Yuen and Garbers, 1992). It is not yet clear whether the formation of heterodimers results in a single functional catalytic unit or if the dimerization simply allows for proper tertiary orientation of the peptide subunits to activate their respective catalytic domains (Fulle and Garbers, 1994). There is also some evidence to suggest that membrane-associated guanylyl cyclases may exist as monodimers to establish their functional catalytic domains. The GC-A isozyme is believed to function as a homodimer, with specific dimerization in the carboxyl terminal domain where the catalytic domain is hypothesized (Thorpe *et al.*, 1991). A possible role for the protein kinase-like domain has been suggested upon the discovery that, in order for atrial natriuretic peptide to optimally activate guanylyl cyclase, the presence of millimolar ATP is required (Chinkers *et al.*, 1991). This concentration of ATP is within the appropriate expected range of intracellular ATP; therefore, ATP may directly modulate the activation of GC-A by ANP. Site-specific mutagenesis has demonstrated that this ATP modulation is associated with the kinase-like domain (Chinkers and Garbers, 1989).

Guanylyl cyclase isozymes can be paralleled to the receptor-G-protein adenylyl cyclase components of the cAMP pathway. Both possess the means through which accumulation of the cyclic nuclectide messengers can be well modulated. The guanylyl cyclase isozymes contain the receptor domain, regulatory domain and catalytic domain (Fulle and Garbers, 1994).

The last several sections have reviewed the components of the cyclic nucleotide pathways, and these are, of course, key sites in the modulation of cyclic

nucleotide accumulation. Alternatively, other than manipulation of the rate and amount of cyclic nucleotide formation, the other means of varying their accumulation is by modulation of the enzymes responsible for their degradation, the phosphodiesterases (PDEs). Since my experiments pertain to changes in the cyclic nucleotide accumulations, it is relevant to briefly review some known properties of PDEs.

I.2.5. Phosphodiesterases

Since cAMP and cGMP initiate a cascade of intracellular events, it is crucial that the intracellular concentrations are tightly controlled. Modulation of cyclic nucleotide synthesis is important in the maintenance of cell homeostasis, but equally important is the regulation of cyclic nucleotide degradation. Phosphodiesterases catalyze the degradation of cAMP and cGMP to their respective 5'-nucleoside monophosphates. Phosphodiesterases not only regulate the steady state (basal) levels of cyclic nucleotide second messengers, but also serve as important modulators of the amplitude and duration of cyclic nucleotide signals (Beavo, 1995).

Mammalian phosphodiesterases include an extensive group of structurally related isozymes, which are further categorized into seven current, distinct though related gene families (Manganiello *et al.*, 1995). It is established that, among the identified mammalian phosphodiesterases, there are many common structural features. Especially well conserved is the C-terminal region of the enzymes (Conti *et al.*, 1991). Most phosphodiesterases are isolated as dimers or multimeric enzyme

complexes, and the dimerizations are typically present in the regulatory domains which are one of the conserved portions (Beltman *et al.*, 1993).

There are seven PDE families, these categorizations correspond to variations in the PDE substrate preference, selectivity and modulation (Manganiello *et al.*, 1995).

The regulation of the various phosphodiesterases is well correlated with phosphorylation events and, as such, lend themselves to modulation by kinases. Variations in phosphodiesterases may be associated with many pathological conditions. Given the critical nature of their function, variations and/or abnormalities in phosphodiesterases are probable candidates for potential cell dysfunction.

Clinical use of phosphodiesterase inhibitors has been implemented in attempts to treat certain pathological conditions associated with phosphodiesterase hyperactivity. The development of numerous selective inhibitors for all of the phosphodiesterase families has been useful, as has the identification of non-selective phosphodiesterase inhibitors, such as 3-isobutyl-1-methylxanthine (IBMX). The latter pharmacological tool has been useful in several of the experiments contained within this thesis, since at millimolar concentrations, it will non-specifically block all phosphodiesterase activity within exposed cells.

The phosphodiesterases isolated from mammalian species share a common generalized structure: especially well conserved is a region of 270 amino acids, which is the domain corresponding to the phosphodiesterases catalytic domain (Conti *et al.*, 1991). This catalytic domain is typically associated with the central

portion of the enzyme between a regulatory domain on its amino terminal side and an extended carboxy end, which as of yet has its function is unknown. There is evidence to support that the cyclic nucleotide phosphodiesterases contain sites that may be responsive to the actions of hormone-induced phosphorylation (Beltman *et al.*, 1993), and the associated role of tyrosine kinases can thus be important in the regulation of cyclic nucleotide metabolism. Therefore tyrosine kinase inhibitors unsurprisingly influence the activities of phosphodiesterases, and likely illicit this effect through interfering with phosphorylation events. The role of phosphorylation/ dephosphorylation as a means of regulating peptide function will be briefly reviewed in a later section.

I.3 Cyclic Nucleotides in the Pineal

Adrenergic regulation of pineal cGMP levels is mediated by both α_1 and β-adrenergic receptors, as is cAMP accumulation (Sugden, 1989). Furthermore, the pinealocyte cGMP accumulation induced by NE treatment requires β-adrenoceptor activation and is potentiated by α_1-adrenoceptor activation (Vanacek *et al.*, 1985, Ho *et al.*, 1987). It appears that intact sympathetic innervation and a normal light-dark cycle are essential for pineal cGMP responsiveness (Klein *et al.*, 1981). Specifically, the cGMP response disappears within a week following pineal sympathetic denervation or constant light (Klein *et al.*, 1981). It has been reported that constant light reduces guanylyl cyclase activity as a result of decreased NO synthase activity (Schaad *et al.*, 1994). On top of the evidence for the presence of

NO-sensitive guanylyl cyclases in the pineal, it has recently been published that there is evidence of the presence of membrane-bound guanylyl cyclase receptors in rat pinealocyte monolayer cultures (Olcese *et al.*, 1994).

I.4. Phosphorylation/Dephosphorylation

Phosphorylation is a post-translational modification used by cells to control the properties of a wide variety of proteins, including enzymes, receptors and ion channels, as well as structural proteins. The true usefulness of protein phosphorylation as a regulatable modification is embodied in its reversibility. It is logical that the steady-state level of phosphorylation of a protein is attributed to the ratio of its rate of phosphorylation and dephosphorylation. It is essential to the usefulness of phosphorylation as a structural modification that modulates protein function; that this ratio of phosphorylation/dephosphorylation is itself modifiable and reversible. These rates are respectively dependent on the intrinsic activities of two classes of enzymes, the cell kinase(s) and phosphatase(s) for the respective protein or amino acid-specific substrate. Protein kinase(s) are phosphotransferases which catalyze the transfer of an active phosphoryl group of ATP to an amino acid side chain in the presence of cofactor ions (i.e. Mg^{2+}). The recipient phosphoryl acceptors include alcohol groups of serine and threonine, or the phenol group of tyrosine residues. Alternatively, phosphatase enzymes are responsible for catalysis of the removal of phosphoryl groups from these amino acid residues, and thus are the antagonist enzymes of the kinase(s). Therefore, if the endogenous activity of an enzyme is

dependent upon its state of phosphorylation/dephosphorylation, the regulation of the balance of its specific kinase and phosphatase enzymes is clearly a reversible mechanism by which a cell can modify its respective enzyme, receptor or channel activities. The kinase enzymes can be subclassified into serine/threonine kinases and tyrosine kinases, depending upon which amino acid phosphorylation they catalyze.

I.4.1. Serine/Threonine Kinases

The serine/threonine kinases represent a large group of cellular kinases which correspondingly have a wide variety of regulatory mechanisms. For instance, protein kinase A is regulated by cAMP levels, protein kinase C is regulated by phosphatidyl serine, diacylglycerol, free fatty acids and calcium (Blackshear *et al.*, 1988; Huang *et al.*, 1993). Additionally, a large group of serine/threonine kinases contain regulatory domains sensitive to Ca^{2+}/calmodulin, the majority of which are thus referred to as CaM kinases. The myosin light chain kinases (MLCK) are also within this group (Hunter, 1995). As would be expected, the serine/threonine kinases retain a conserved catalytic domain (Girault, 1994).

The serine/threonine kinases have been demonstrated to directly induce gene promotors, as well as the activities of other cellular proteins, and thus serve as an important mediators of cell response. One of the important substrates of PKA, for example, is the transcriptional factor called cAMP response element-binding protein (CREB) (Hunter, 1995). CREB binds to a cAMP-regulated enhancer region

in cAMP-inducible genes. PKA phosphorylates a serine residue within the CREB moiety which significantly promotes its DNA binding activity so as to induce transcription of cAMP-responsive genes (Hagiwara *et al.*, 1993). This example illustrates how the elevation of cellular cAMP accumulation can result in initiation of a physiological response by the cell, in this case an increase in gene transcription. Though not all serine/ threonine kinase activity results in transcriptional events, others are involved in receptor down-regulation, channel regulation and a myriad of other cellular responses.

Another important class of serine/threonine kinases is the PKCs, which are not only activated via cell-hormone interaction, but also translocated to various cell surfaces. Prior to cell stimulation, PKC is present in the cytosol, and when the cell is stimulated with either phorbol ester, calcium, or PIP_3, or hormones that elevate intracellular diacylglycerol, the PKC is translocated to various membranes throughout the cell (Mochly-Rosen, 1995). In fact, individual activated PKC isozymes are differentially compartmentalized, which can be seen to suggest that they mediate distinct cellular functions within the realm of their translocated sites (Muhl and Pfeilschifter, 1994). The roles of the various PKC isozymes are not well characterized, but they have been localized to many specific brain regions and certain specific cell types, which suggests that they regulate specific cellular functions. Both α and β isozymes are known to be present within the pineal gland; therefore, the PKC family likely plays an important role in the regulation of at least some aspect of pinealocyte function (Blackshear *et al.*, 1988). The activation of PKC

is usually associated with cell multiplication, and the action of many oncogenes involves an abnormal hyperactivation of PKC (Girault, 1994). However PKC is also found in cells that do not multiply, like pinealocytes for instance, and can be associated with numerous cell functions, such as the modulation of guanylyl cyclase. Current research is attempting to better understand all aspects of the PKC pathway, both in normal and abnormal cell states, as it is obviously of considerable importance to the maintenance of cell homeostasis.

Although there are many other examples of serine/threonine kinase which are involved in a correspondingly broad array of cellular responses and regulatory mechanisms, the tyrosine kinase family is more relevant to my thesis work. Before completing this brief introduction to the serine/threonine kinases, it is important to emphasize that the action of the serine/threonine kinases are intertwined with the functions of other transduction processes, including those of the tyrosine kinases. For instance, it has been found in β lymphocytes that activation of phospholipase C is associated with tyrosine phosphorylation, and since PLC activation increases $[Ca^{2+}]_i$ and DAG, which activate PKC, this is an example of association of tyrosine kinase and serine/threonine kinase pathways (Padeh *et al.*, 1991; Roifman and Wang, 1992). Correspondingly, erbstatin and tyrphostins, tyrosine kinase inhibitors block protein serine kinase activation in sea star oocytes (Daya-Makin *et al.*, 1991). It has further been suggested that PKC could reduce tyrosine phosphorylation in rat pinealocytes (Ogiwara *et al.*, 1997). This suggestion is supported by the studies demonstrating PKC mediated phosphorylation of EGF and insulin receptors

resulting in a reduction in their respective tyrosine kinase activities (Cochet *et al.*, 1984, Takayama *et al.*, 1988).

I.4.2. Tyrosine Kinases

The tyrosine kinase group is sometimes subdivided into the non-receptor tyrosine kinases and the receptor tyrosine kinases. Both of these groups of tyrosine kinase peptides have their place in the functional aspects of cellular signal transduction processes. The receptor protein tyrosine kinase (RPTK) family is involved in the control of cell growth, differentiation and metabolism. The RPTKs are activated by polypeptide ligands which are specifically referred to as growth factors or cytokines. The defining component of the RPTKs is their intrinsic protein tyrosine kinase activity that is regulated specifically via their interaction with their respective ligands. RPTKs contain many common structural features, such that a description of a generic RPTK can be established. The N-terminus of the RPTK is outside the cell and has a signal sequence which targets the protein for membrane insertion (Girault, 1994). Also on the extracellular portion of the peptide, one or more cysteine-rich regions are found which serve as the ligand-binding domain for the RPTK (Hunter, 1995). The transmembrane domain consists of a sequence of hydrophobic residues. The catalytic domain is located on the cytoplasmic side of the cell and is a well conserved domain that is not only observed in the non-receptor tyrosine kinase group, but also in the protein-serine/threonine kinases (Blackshear *et al.*, 1988). This catalytic domain is typically about 250 amino acids, with compara-

tive homologies as high as 95% between RPTKs (van der Geer *et al.*, 1994).

The RPTKs are known to be the receptors for insulin, epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and many other peptide hormones (van der Geer *et al.*, 1994). For simplicity, a brief description of the insulin receptor tyrosine kinase will be used to represent the typical RPTK. The function of a given RPTK can be attributed to the specific cells where it is expressed and the amount of receptor expressed. Additionally, the RPTK function is dependent upon its affinity for its ligand and the existence of the given cells regulatory processes.

I.4.3. The General Tyrosine Kinase Receptor

The insulin receptor was first shown to have the capacity of autophosphorylation in the early eighties, and that this autophosphorylation was associated with its tyrosine kinase activity (Rosen *et al.*, 1983), since then this has been recognized as a common property of all RPTK's. Subsequent to the primary amino acid sequence analysis, several possible sites for this regulatory autophosphorylation were discovered. Specifically, thirteen tyrosine residues located in the cytoplasmic portion of the subunit of the insulin receptor were described as candidates for tyrosine autophosphorylation (Blackshear *et al.*, 1988). *In vitro* studies revealed that at least six of these tyrosine residues were autophosphorylated and, perhaps more importantly, that this phosphorylation was stimulated by insulin treatment (Tornquist *et al.*, 1988). Therefore, this was direct evidence showing how a RPTK interacts

with its ligand and is subsequently activated by its own autophosphorylation of specific tyrosine residues. There has been some evidence that the term autophosphorylation is a misnomer in that the formation of receptor dimers and reciprocal phosphorylation might be the actual means of tyrosine phosphorylation (van der Geer *et al.*, 1994). What is essential is the evidence supporting ligand activation of receptor tyrosine kinase activity via tyrosine phosphorylation. Furthermore, it is important to remember that the activation of an RPTK via its interaction with its ligand is a result of tyrosine phosphorylation, which is a transient event, allowing for the activation to be of regulatable amplitude and duration (Hunter, 1995). Once activated, the RPTKs can influence a number of cellular peptides via their ability to phosphorylate tyrosine residues, therefore the downstream effects of RPTK activation influence many signalling pathways such as phosphlipase C, mitogen-activated protein kinase and others. It is within the past decade that a better understanding of how this interaction between an activated receptor may interact with another substrate peptide via an Src homology domain (Blackshear *et al.*, 1988). Src Homology domain, SH_2 domain will be briefly discussed following a short introduction to non-receptor tyrosine kinases, also referred to as soluble tyrosine kinases. Before the next brief review, it may be useful to refer to Fig. I.3. showing the structure of various RPTKs so as to illustrate how the description of the insulin receptor might be similar to other RPTKs on the basis of conserved domains.

I.4.4. Non-Receptor Tyrosine Kinases

As was reviewed earlier, the catalytic domain of the non-receptor tyrosine kinases is a conserved domain with many homologies to the RPTK catalytic domain (Blackshear *et al.*, 1988). The first protein tyrosine kinase to be discovered, and perhaps the most well characterized, was a product of the Rous sarcoma virus oncogene, named "v-src" (Ogawa *et al.*, 1994), and I will utilize this in my general review of non-receptor tyrosine kinases. The discovery and comparison of v-src with a homologous tyrosine kinase with different activities, c-src, has assisted in clarifying how these cytosolic tyrosine kinases are regulated (van der Geer, 1995). Structural comparisons between these two kinases showed that, in c-src, 19 amino acids in the carboxyl terminus are replaced with a different sequence of twelve amino acids (Blackshear *et al.*, 1988). Additionally, the phosphorylation sites were also quite different, such that *in vivo* v-src was typically phosphorylated at tyrosine residue 416, whereas c-src was phosphorylated at tyrosine residue 527, for which there is no structural homologue in v-src (Snyder *et al.*, 1983). Point mutation studies have assisted in the clarification of the significance of these two tyrosine sites with regard to the protein kinase activity of the v-src and c-src peptides. The conclusions drawn were that phosphorylation of src kinase *in vivo* at tyrosine residue 416 (Kmiecik *et al.*, 1987) is important in the activation of the intrinsic kinase activity and, additionally, phosphorylation of site 527 in c-src conversely reduces the tyrosine kinase activity (Hunter, 1987). Therefore, phosphorylation of tyrosine residues within the cytosolic protein tyrosine kinase moiety is important in the regulatory activity, similar to that which was observed in the RPTKs. This is not sur-

prising, given the structural similarities between the RPTKs and the non-receptor tyrosine kinases. The understanding that both classes of tyrosine kinases are themselves regulated through phosphorylation events, substantiates the importance of phosphorylation events on cellular function. Fig. I.3 shows the generalized structure of several non-receptor tyrosine kinases (Girault, 1994).

Another important region which is conserved within the family of src kinases has been described as the src homology 2 domain (SH_2). Realization that this SH_2 domain is observable in many of the tyrosine kinase proteins and other cellular peptides has promoted a plethora of research into the possibility of this domain serving a functional importance in kinase substrate recognition. The following section will pertain to some of the current literature published on the significance of SH_2 domains.

I.4.5. SH_2 Domains

SH_2 domains are about 100 amino acid residues in length and were first identified in the src kinase, outside the catalytic domain (van der Geer *et al.*, 1994). The significance of these conserved domains was clarified by the finding that SH_2 domains bind specifically to phosphorylated tyrosine residues (Mayer *et al.*, 1991) and that this binding was of high affinity. Subsequent analysis has demonstrated that the SH_2 domain binding affinity is a result of the interaction of the phosphotyrosine with a pocket containing lysine and arginine residues (Pawson and Gish, 1992). Also important is the interaction of amino acid residues directly on the C-

terminal side of the phosphorylated tyrosine residues, and these residues establish variations in selectivity for specific SH_2 domains (Fantl *et al.*, 1992). It is well documented that SH_2 domain-containing proteins have the ability to bind phosphorylated tyrosine residues associated with both the RPTKs and the non-receptor tyrosine kinase family, which implicates SH_2 as regulators of protein-protein interactions in these signal transduction processes (Clark *et al.*, 1992). Support for this discovery is added by the identification of SH_2 conserved domains in a diverse group of cytoplasmic signalling proteins.

The RPTKs are able to interact with the SH_2 domain containing proteins following their own ligand-induced autophosphorylation. This binding of RPTK to SH_2 domains can facilitate tyrosine phosphorylation of the SH_2-containing peptide (van der Geer *et al.*, 1994). Alternatively, binding of SH_2-containing peptides with phosphotyrosine groups may allosterically affect that protein's activity and may also serve as a mechanism of localizing the SH_2-containing peptide to a particular site within the cell. Therefore, SH_2 domains physiologically function to increase specificity, efficiency and localization of the substrates of tyrosine kinase enzymes.

With the accumulation of evidence that tyrosine kinase activities are important to the actions of many growth factors, and that in hyperactivity, tyrosine kinase is often associated with neoplasias (Maminta *et al.*, 1992), the investigation into the effects of tyrosine kinase inhibitors became an important area of research.

I.5. Tyrosine Kinase Inhibitors

Enhanced tyrosine kinase activity has been implicated in both malignant proliferative diseases and in non-malignant proliferative diseases, such as atherosclerosis and psoriasis (Levitzki and Gazit, 1995). Therefore, development of tyrosine kinase inhibitors may be quite useful physiological and pharmacological tools for the study of the tyrosine kinase signal transduction pathway and the treatment of pathological conditions associated with them, respectively. The potential utilization of PTK inhibitors as antiproliferative drugs was suggested as early as 1981, when quercetin was used to suppress protein kinase activity in tumour cells (Graziani *et al.*, 1981). Quercitin was the first natural protein tyrosine kinase blocker discovered, but it also inhibits other ATP-requiring enzymes, such as PKA and PKC. Another flavone compound, genistein, has been shown to be somewhat more selective towards PTKs than quercitin, but is also functions via competitive inhibition with ATP (Gazit *et al.*, 1989). In the attempt to synthesize PTK inhibitors that competitively inhibit the PTKs through a mechanism not directly involving ATP, several compounds were generated from the benzylidenemalononitrile (BMN) nucleus (Gazit *et al.*, 1989). This family of synthetic PTK blockers was named the tyrphostins and are the primary pharmacological tool used in this research project.

I.5.1. Tyrphostins

As various synthetic compounds were generated and tested for their respective efficacy of inhibiting EGF receptor kinase activity, the introduction of cyano-, keto- and amide- groups was found to be beneficial with regard to tyrphostin poten-

cies (Levitzki, 1992). Tyrphostin B42, N-benzyl-3,4-dihydroxybenzylidene cyano-acetamide is the tyrphostin compound that is the focus of my research project and its structure is shown with that of tyrphostin A1, 4-methoxybenzylidene malonitrile as a comparison (Fig. I.4). The use of tyrphostin A1 in my experiments serves as a compound of similar structure to tyrphostin B42, that is shown to be a less effective tyrosine kinase inhibitor. Therefore when assumptions are made suggesting observed effects of tyrphostin B42, and these effects are correlated with tyrosine kinase inhibition, support is conferred by an observation of smaller or negligible effects with tyrphostin A1 treatment.

Various tyrphostins have been used in the treatment of a wide array of cell types as tools to reveal the correlations between tyrosine kinase activity and the actions of growth factors and signal transduction pathways. For instance, tyrosine phosphorylation is an essential component of the actions of many growth factors, and tyrphostin treatment has been shown to inhibit mitogenic actions of PDGF (Bilder *et al.*, 1991; Seckl and Rozengurt, 1993). Since PDGF is believed to be significant in the development of vascular smooth muscle disease, such as athero-sclerosis, the inhibition of PDGF-induced DNA synthesis in smooth muscle cells by tyrphostin may be of clinical relevance (Bilder *et al.*, 1991). Tyrphostins have further been shown to inhibit cell functions mediated by follicle-stimulating hormone (FSH) (Gomberg-Malool *et al.*, 1993; Orly *et al.*, 1994), nerve growth factor (Ohmichi *et al.*, 1993), epidermal growth factor (Yaish *et al.*, 1988; Lyall *et al.*, 1989; Osherov *et al.*, 1993; Miyaji *et al.*, 1994; Stephan and Dziak, 1994) and many other peptide

hormones. These are just a few examples demonstrating the significance of tyrosine phosphorylation on the mechanisms of action of numerous peptide hormones, and how a tyrphostin can influence the signalling induced by these peptides in cells.

Additionally, tyrphostins have been key tools, used in conjunction with other tyrosine kinase inhibitors, to establish cell processes that utilize tyrosine kinase activity to orchestrate cell responses. Tyrphostins were utilized in the demonstration that β-lymphocyte (Padeh *et al.*, 1991) and T-lymphocyte activation (June *et al.*, 1990) are dependent upon protein tyrosine phosphorylation, implicating tyrosine kinase enzymes as an essential element in these respective cell signal transduction pathways.

Other studies have discovered the involvement of tyrosine phosphorylation in the generation of other second messengers as a result of the utilization of tyrphostins as pharmacological tools. For instance, the accumulation of nitric oxide can be suppressed via tyrphostin treatment in neuronal and glial cells (Feinstein *et al.*, 1994; Rodriguez *et al.*, 1994), as well as in macrophages (Dong *et al.*, 1993). Tyrphostins also inhibit inositol triphosphate (IP_3) production and action (Conti *et al.*, 1991) in pancreatic cells (Piiper *et al.*, 1994) and have also been shown to attenuate intracellular Ca^{2+} accumulation (Lee *et al.*, 1993; Yule *et al.*, 1994). Other studies have illustrated significant associations between tyrosine kinase-linked pathways with G-protein pathways, such as the influence of EGF receptor kinase on cardiac adenylyl cyclase (Nair *et al.*, 1993). Given the diversity of cellular physiologic responses that are affiliated with the phosphorylation of tyrosine, be

they induced through cell exposure to peptide hormones, or other agents, the potential of tyrphostins as an investigative tool is proportionately diverse. There are a multitude of diseases associated with hyperactivity or hyper-expression of tyrosine kinases which may be targets for tyrphostin drug implementation (Levitzki and Gazit, 1995).

Given the multitude of different tyrphostins commercially available an initial screening of several different tyrphostin compounds was performed resulting in the selection of the most potent tyrphostin tested, tyrphostin B42. Conversely the low potency tyrphostin A1 was selected as a control comparison for my subsequent experiments, as is typical in many tyrosine kinase inhibitor studies.

I.6. Specific Aim of the Present Study

The rat pineal gland and cultured rat pinealocytes are good models for examination of signal transduction pathways, and further represent a model for a functional endocrine gland or cell, respectively. It has been demonstrated that the accumulation of cAMP stimulates the activity of serotonin N-acetyltransferase, which is the rate-controlling enzyme for the pineal's synthesis of its hormone, melatonin (Deguichi and Klein, 1973; Roseboom and Klein, 1995). The regulation of the pineal gland via innervation by sympathetic ganglia suggests that adrenergic stimulation is at least one mechanism by which the pinealocyte activity may be modulated. It has been demonstrated that adrenergic regulation of cGMP is mediated by both α_1 and β adrenergic receptors in the plasma membrane of

pinealocytes (Vanecek *et al.*,1985; Sugden *et al.*, 1989). Similarly, cAMP accumulation within pinealocytes is also elevated in response to adrenergic stimulation (Vanecek *et al.*,1985 ; Ho *et al.*, 1987). The concentrations of cyclic nucleotide accumulation within cells is a product of the balance of cyclic nucleotide synthesis and cyclic nucleotide degradation. Therefore, the regulation of the components of the cyclic nucleotide synthesis pathways, as well as the regulation of phosphodiesterase activities, are critical to the endogenous activities of the pinealocytes that are regulated via cyclic nucleotide second messengers.

It has recently been reviewed that one of the key mechanisms through which intracellular receptors, channels, modulator peptides and enzymatic peptides are regulated is through phosphorylation events (van der Geer *et al.*, 1994). Additionally, many of the mechanisms through which second messengers themselves induce physiological changes in response to signals is through activation of kinases and/or phosphatases. More specifically, the phosphorylation of tyrosine residues is known to be a primary mechanism in the activation sequence of growth factor receptors and cytosolic kinases.

When considering the significance of both cyclic nucleotides and phosphorylation of tyrosine residues in the regulation of cell function, it was hypothesized that tyrosine phosphorylation may play an important role in cellular cyclic nucleotide accumulation. It is well established that tyrosine kinase is important in general signal transduction processes, as in receptor tyrosine kinases. Even more specifically it is established that tyrosine kinases are important in the regulation of G-protein

coupled signal transduction, as is documented for G-protein coupled receptor kinases (Premont *et al.*, 1995). It is also documented that tyrosine kinase activity is a mechanism for the activation of PKC such that it has been inhibited in platelet cells using genistein as the tyrosine kinase inhibitor (Dhar *et al.*, 1990). Furthermore, PKC is known to be involved in the pineal cAMP accumulation (Sugden *et al.*, 1985), and pineal cGMP accumulation (Ho and Klein., 1987). More recently, it was shown that in pinealocytes, pituitary adenylate cyclase-activating polypeptide (PACAP) increases cAMP accumulation in association with α_1-adrenergic and PKC mechanisms that fail to elevate cGMP accumulation (Chik and Ho, 1995). This result demonstrates a difference in pineal cAMP and cGMP regulation. To summarize, it is apparent that tyrosine kinase activity influences cellular signal transduction, including G-protein-coupled pathways. Tyrosine kinase also influences PKC activity, and PKC is known to be involved in cyclic nucleotide regulation in cells, including pinealocytes. It was not known whether tyrosine kinase activity influenced cyclic nucleotides in pineal cells. Hence, it became relevant, and the objective of this thesis, to determine whether tyrosine kinase activity in pinealocytes influenced cyclic nucleotide accumulation. Therefore, with the availability of the tyrphostins, protein tyrosine kinase inhibitors, we decided to investigate if a relationship between cyclic nucleotide accumulation and tyrosine phosphorylation was present in rat pinealocytes. Specifically, this research project was designed to investigate (1) the effect of tyrphostin B42 on the accumulation of cAMP and cGMP in rat pinealocytes; (2) the effect of tyrphostin B42 on the accumulation of cyclic

nucleotides in cultured rat pinealocytes stimulated with isoproterenol/prazosin and norepinephrine; (3) the site(s) of action of tyrphostin B42 with regard to its effect on the pineal cyclic nucleotide accumulations; (4) the effect of tyrphostin B42 in the presence of activators of the cAMP synthetic pathway, cholera toxin and forskolin; (5) whether tyrphostin B42 resulted in the modulation of cyclic nucleotide synthesis and/or degradation; and (6) whether the observed effects of tyrphostin B42 were the same for cAMP accumulation and cGMP accumulation.

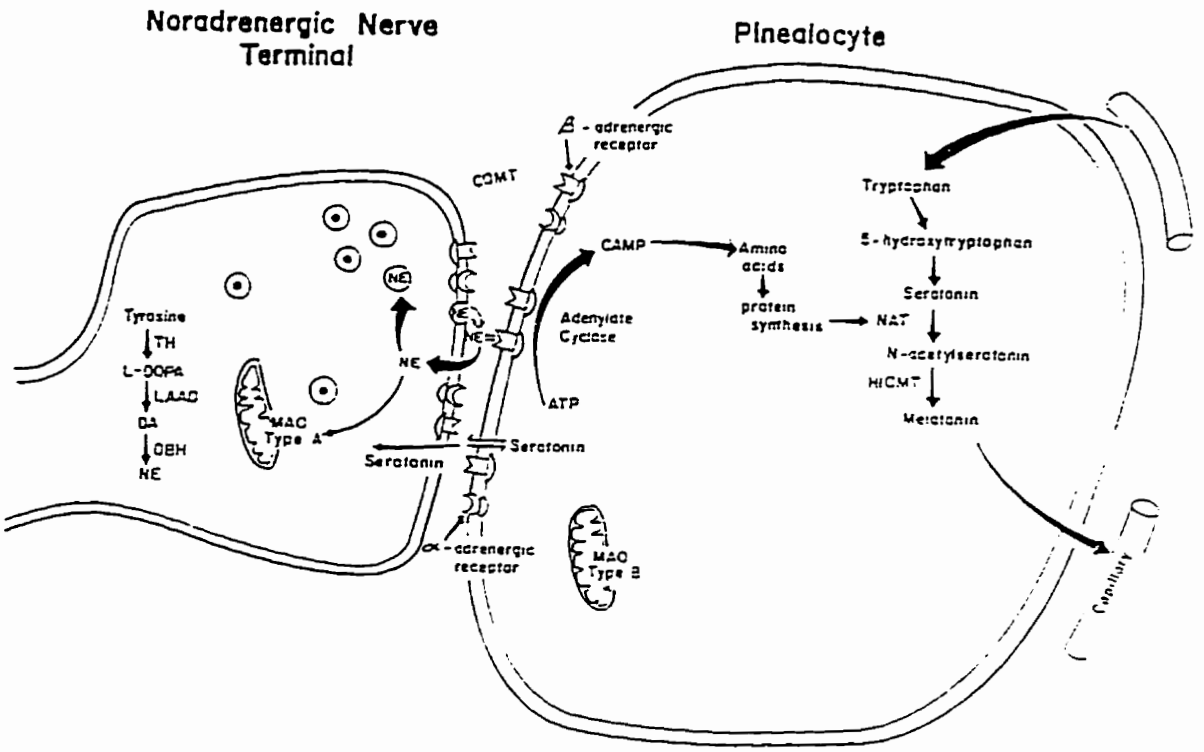


Figure I.1. A schematic representation of the sympathetic innervation of pinealocytes and the receptor-activated intracellular metabolism that is influenced by norepinephrine. A β adrenergic receptor is coupled by a G-protein to adenylyl cyclase that converts ATP to cAMP. Cellular mechanisms for protein synthesis are activated by cAMP-dependent reactions, elevating levels of N-acetyltransferase and, thereby, the synthesis of melatonin. An α_1 adrenergic receptor that is coupled via a G-protein to phospholipase C is activated also. α_1 adrenergic receptor activation potentiates the accumulation of cAMP and the synthesis and release of melatonin. (from Lolley *et al.*, 1992, Neurochem. Res. 17(1): 85.)

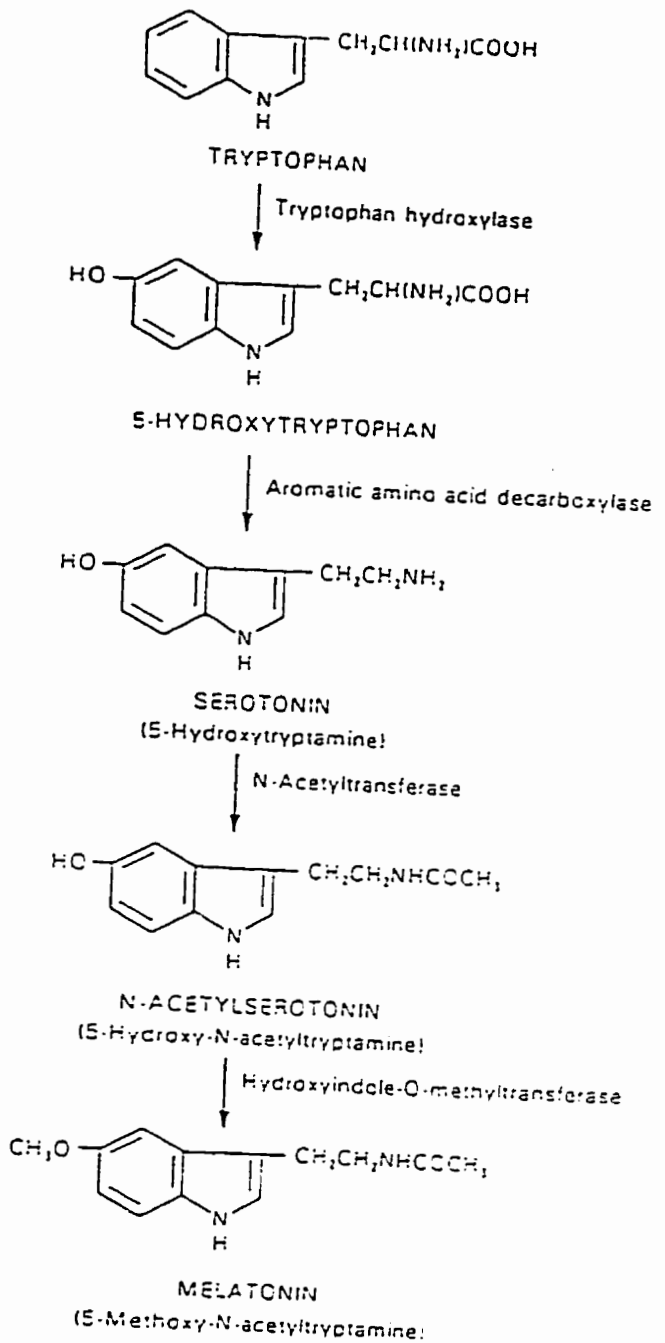
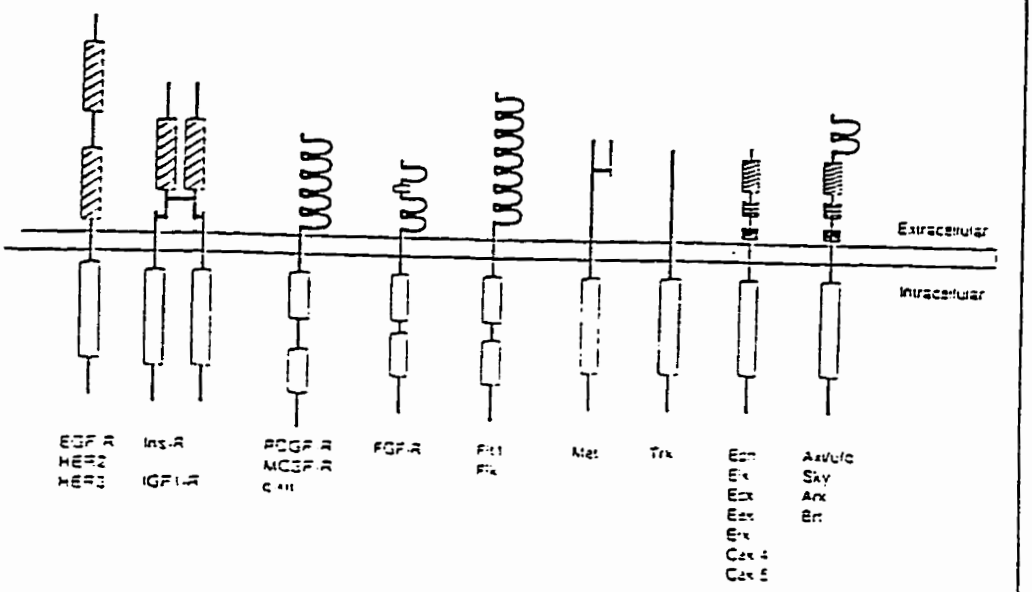


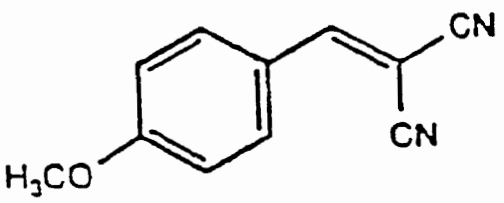
Figure I.2. The conversion of tryptophan to melatonin. (from Klein, 1985. CIBA Symp. 117: 40.)



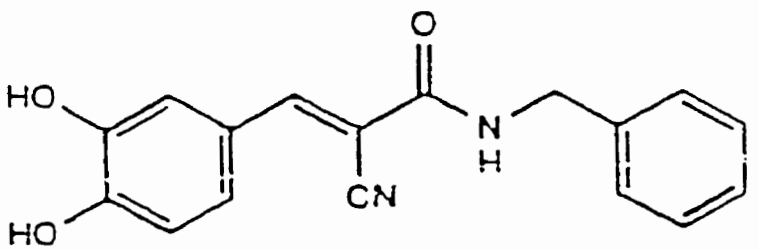
Structure of receptor tyrosine kinases.

Conserved domains include the intracellular catalytic domain (stippled) which can be interrupted by a short sequence (kinase insert), and various extracellular domains: cysteine-rich (striped), immunoglobulin-like (circles), acid box (open), fibronectin type III (horizontal stripes). The amino terminus is extracellular. For many of these receptors the ligands are unknown. After Fantl et al. *Ann. Rev. Biochem.* 62, 453–481 (1993).

Figure I.3. Structure of receptor tyrosine kinases. (from Girault, 1994, Neurotransmissions X(3): 1-6.)



Tyrphostin 1



Tyrphostin B42

Figure I.4. Chemical structure of tyrphostin A1 and tyrphostin B42.

α Subunits	β Subunits
K^+ channel (I_{KAch})	K^+ channel
K^+ channel (K_{KATP})	-
Adenylyl cyclase I	Adenylyl cyclase I
Adenylyl cyclase II (IV)	Adenylyl cyclase II (IV)
Adenylyl cyclase III	-
Phospholipase C 1	Phospholipase C 1^b
Phospholipase C 2	Phospholipase C 2
-	Receptor kinases (-adrenergic, muscarinic
cGMP Phosphodiesterase	-
-	Phospholipase A_s
Calcium channel	Calcium channel (?)
-	Yeast pheromone response pathway

Table I.1. Effectors regulated by G protein subunits (modified from Conklin and Bourne, 1993, Cell 73: 632).

Regulatory entity	Mode of regulation	Adenylyl cyclase types*	Comments
G_s-α	Stimulatory	1-6	
G_i-α	Inhibitory	2,3,6	All types may be inhibited Inhibition could be direct
G-βγ	Direct stimulation in the presence of α_s	2, 4	Type 7 may also be stimulated because it is like 2 and 4
	Direct inhibition	1	
	No direct effect	3, 5, 6	
Forskolin	Stimulatory	1-6	Forskolin binds directly to type 1-6 adenylyl cyclases
Ca^{2+}/CaM	Stimulatory	1, 3	CaM binds directly to type 1 enzyme
Ca^{2+}	Inhibitory**	5, 6	Effect is not through CaM; probably directly on the enzyme
Adenosine (P-site ligands)	Direct inhibition	1, 5, 6	Though types 2, 3 and 4 have not been tested, they are also likely to be inhibited
Protein kinase C	Stimulation	2	Smaller stimulatory effects are also seen on type 1 and 3 enzymes. Protein kinase C suppresses α_s inhibition of type 2
Protein kinase A	Inhibition	6	Direct effect not yet reported

* Currently no functional properties of types 7 and 8 adenylyl cyclases are known, because full-length functional clones have not been maintained and expressed

** This refers to inhibition at low (μM) Ca^{2+} concentrations. At higher (~100 μM) concentrations, all adenylyl cyclases are inhibited like other Mg-dependent enzymes

Table I.2. Regulation of various adenylyl cyclases (modified from Tang and Gilman, 1992, Cell 70: 869).

II. Materials and Methods

II.1. Cell Culture

Male Sprague-Dawley rats (180 to 200 g) were decapitated and the pineal bodies were excised and collected in ice-cold phosphate buffered saline (Appendix 1). The pineal stock was removed and the glands were then washed three times with phosphate buffered saline (PBS). The pineal glands were then transferred to a digestion medium consisting of Dulbecco's Modified Eagle Medium (DMEM), trypsin (1 mg/ml) and DNAse (0.01%). The glands were incubated in a 37°C water bath while shaking for 8 min. The trypsinization reaction was then stopped via a quenching addition of fetal calf serum (FCS). The solution was centrifuged for 8 min at 650 g. The supernatant was aspirated and discarded, while the pellet was resuspended in PBS. The cells were then triturated using a glass pipette to complete the cell dissociation. The cells were then recentrifuged for 8 min at 800 g, and the supernatant was aspirated and discarded. The pellet was resuspended in DMEM supplemented with 10% FCS. The cells were subsequently counted and evaluated for viability using the trypan blue dye exclusion method. A typical cell count of about 4.5 million viable cells was obtained from 25 excised pineal glands. The pinealocytes were then incubated in a humidified 5% CO_2 incubator at 37°C overnight.

The following day, the pinealocytes were washed three times in DMEM with FCS and were aliquoted into microcentrifuge tubes at a known cell concentration,

which was typically 20,000 cells per 400 µl DMEM with FCS. The aliquoted cells were allowed an hour stabilization prior to administration of experimental drugs.

II.2. Drug Preparation

Isoproterenol, norepinephrine and cholera toxin were prepared in solutions of distilled and deionized water, whereas tyrphostins and forskolin were prepared in a solution of dimethyl sulfoxide (DMSO). If multiple drugs were being utilized which were in DMSO solutions, their respective concentrations were adjusted such that the DMSO concentration within the test microcentrifuge tube was minimized to < 1% , or a DMSO control was created at the same comparable concentration.

II.3. Drug Treatment

The pinealocytes were aliquoted into microcentrifuge tubes at a concentration of 16,000 to 25,000 per tube. Each test group comprised four of these tubes such that quadruplicate samples were generated for the drugs. The typical drug exposure time was 15 min; however, some drugs required pretreatments or longer incubation times, which are indicated. All incubations were at 37°C in a humidified, dark, 5% CO_2 atmosphere incubator. After the drug exposure and incubation, the microcentrifuge tubes were spun for 3 minutes and the supernatant was aspirated and discarded, leaving the pinealocytes as a small pellet. Each tube received 100 µl cold 5 mM acetic acid solution, to lyse the pinealocytes, and was frozen over dry ice. The tubes were then boiled for 5 min in a water bath so as to prevent the

bacterial degradation of the cyclic nucleotides prior to the assay.

II.4. Radioimmunoassay Detection of Cyclic Nucleotides

After the pinealocytes were lysed in 5 mM acetic acid and alternating freezing and thawing, the samples were ready for cyclic nucleotide radioimmunoassay (RIA) analysis. The cyclic nucleotide assay protocol requires that the samples are acetylated prior to the implementation of the double antibody RIA protocol (Harper and Brooker, 1975).

Standard curves, ranging from 2,000 to 2 fmole known concentration, were generated for cAMP and cGMP in glass test tubes. Separate assay tubes were set up for each sample for analysis of cAMP and cGMP accumulation. An aliquot of 40 µl was taken from each sample for cAMP and the same amount for cGMP sample analysis. To all the sample and standard test tubes, sodium acetate buffer (NaAc; see Appendix) was added in conjunction with normal rabbit serum and I^{125} radiolabelled ligand. Additionally, all test tubes, other than the "Total" and the "Non-specific binding (NSB)" tubes, received the appropriate first antibody. The tubes were then covered and incubated at 4°C overnight in a laboratory refrigerator.

The next day, each tube, except the "Total", received a 50 µl aliquot of the second antibody, sheep anti-rabbit (SAR). These tubes were subsequently incubated under 4°C refrigeration for a minimum of 1 hr. After this incubation period, polyethylene glycol was added to all tubes except the "Total", and those tubes were centrifuged at 3000 g for 45 min at 4°C. The supernatant was decanted and dis-

carded, and the pellet precipitate was counted using a gamma counter (Cobra Auto-Gamma, Canberra Packard, Mississauga, Ontario). The counts were then converted into a value of pmoles cyclic nucleotides per 100,000 cells, via a computer extrapolation in fmoles as compared to the standard curve. The value for each tube in fmoles was multiplied by a correction factor determined by the proportion of sample volume used, the number of cells, and the unit conversion from femto- to picomoles.

II.5. Statistical Analysis

The data presented are those of means ± the standard error of the mean (SEM) of the amount of cyclic nucleotide in a minimum of four replicates of cell samples. The experiments were repeated at least twice with cells from different cell preparations to verify that the results obtained were consistent. The statistical stringency was placed at $p < 0.05$ using a two-tailed unpaired *t*-test. The final results were expressed in pmoles/100,000 pineal cells.

II.6. Materials

Trypsin, DNAase, albumin and trypsin inhibitor for the cell preparations were obtained from Sigma Chemical Corp. (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) was purchased from Gibco (Grand Island, NY). Cholera toxin, isobutylmethylxanthine, fetal bovine serum, isoproterenol, norepinephrine and dimethyl sulfoxide were obtained from Sigma Chemical Corp. (St. Louis, MO). Forskolin,

tyrphostin B42, tyrphostin A1, tyrphostin A25, tyrphostin B44 and tyrphostin B48 were obtained from Calbiochem (La Jolla, CA).

Antibodies for the RIA of cAMP and cGMP were gifts from Dr. A. Baukal (National Institutes of Health, Bethesda, MD). ^{125}IcAMP and ^{125}IcGMP were obtained from ICN (Costa Mesa, CA.).

	Tyrphostin Cross-Reactivity
A1	< 0.002%
B42	< 0.002%
A25	< 0.002%
B44	< 0.002%
B48	< 0.002%

Table II.1. Cross reactivity of tyrphostins with RIA of cAMP/cGMP

III. RESULTS

III.1. Effect of Tyrphostin B42 on cAMP Accumulation

a. Effect of Tyrphostin B42 Alone on cAMP Accumulation

Tyrosine kinase inhibitors have been shown to influence intracellular levels of cAMP in a variety of cell types (Ohno *et al.*, 1993). In this study, the effect of tyrphostin B42 on intracellular cAMP accumulation in male rat pinealocyte cells was examined and measured using radioimmunoassay and comparison to standard curves generated using known cyclic nucleotide concentrations (Fig. III.1). The basal cAMP level measured in rat pinealocytes was .05±.01 pmoles/100,000 cells. Although addition of tyrphostin A1 (300 μM) did not result in a significant change in the cAMP accumulation, it was observed that treatment with tyrphostin B42 (300 μM) caused a significant elevation in the measured cAMP accumulation. The cAMP accumulation measured subsequent to 28 min of pinealocyte exposure to tyrphostin B42 (300 μM) treatment was .21±.02 pmoles/100,000 cells. This is a moderate increase in cAMP accumulation, but was demonstrated consistently over repeated experiments (n>4). Furthermore, this is a different result than that published for another structurally different tyrosine kinase inhibitor, genistein, which did not increase pinealocyte cAMP accumulation significantly above basal levels (Ogiwara *et al.*, 1995).

b. Effect of Tyrphostin B42 on Isoproterenol/Prazosin-Stimulated cAMP Accumulation

Isoproterenol is an adrenergic agonist, established as a means of stimulating cAMP production. When used in combination with prazosin, it serves as a specific β-adrenoceptor ligand. Through increases in cAMP accumulation, pinealocytes are able to respond to adrenergic ligand interaction. Therefore, this study assessed the effect of tyrphostins on the adrenergically-initiated increase in cAMP accumulation (Fig. III.1). Exposure of pinealocytes to isoproterenol/prazosin (1 μM) for 13 min resulted in an increase in pinealocyte cAMP accumulation to a significant extent ($p<.05$) above the basal pinealocyte cAMP level. β-adrenergic activation of pinealocytes resulted in a measured cAMP accumulation of 2.91±.16 pmoles/100,000 cells. Treatment of the adrenergically-stimulated pinealocytes in combination with tyrphostin resulted in further significant increases in measured cAMP accumulations. The pinealocyte exposure to tyrphostin A1 (300 μM) and adrenergic stimulation resulted in a measured cAMP accumulation of 5.85±.81, and an even larger increase induced by tyrphostin B42 (300 μM) measured as 9.68±1.45 pmoles/100,000 cells. Therefore, tyrphostin B42 increases the cAMP both on its own above basal levels, and also increases the isoproterenol-stimulated cAMP accumulation in rat pinealocytes. The observed increase in cAMP accumulation can be attributed to either an increase in cAMP synthesis, a reduction in cAMP breakdown, or a combination of both.

III.2. Effect of Tyrphostin B42 on cGMP Accumulation

a. Effect of Tyrphostin B42 Alone on cGMP Accumulation

The measurement of cGMP in pinealocytes paralleled the cAMP determinations; therefore the pretreatment time and the incubation times were the same (Fig. III.2). The basal pinealocyte cGMP accumulation was determined to be 0.05±.01 pmoles/100,000 cells, and treatment of pinealocytes with tyrphostin B42 resulted in a dramatic increase in the measured cGMP accumulation. Following a 28 min incubation of the pinealocytes exposed to tyrphostin B42 (300 μM), the cGMP content was determined to be 1.89±.16 pmoles/100,000 cells. It is evident that this increase is substantially larger in proportion to that previously cited for cAMP. Moreover, the large significant (p<.05) stimulation observed in the cGMP accumulation induced by tyrphostin B42 suggests that pinealocyte basal cGMP is possibly modulated to a large extent by tyrosine kinase activity. Further support for these results was published for other mechanistically and chemically distinct tyrosine kinase inhibitors, such as genistein and erbstatin (Ogiwara *et al.*, 1995). Again the observed increases in cGMP as with cAMP may be attributed to either an increase in synthesis or a decrease in metabolism , or a combination of the two processes.

b. Effect of Tyrphostin B42 on Isoproterenol/Prazosin-Stimulated cGMP Accumulation

The activation of the pinealocyte β-adrenergic pathway through treatment

with isoproterenol/prazosin (1 μM) elevated the cGMP accumulation about ten-fold above the basal cGMP to 0.64±.10 pmoles/100,000 cells (Fig.III.2). This stimulation of cGMP accumulation was shown to be further potentiated through treatment with tyrphostin B42 (300 μM), such that the measured cGMP was 3.32±.19 pmoles/ 100,000 cells. These results illustrate that treatment of pinealocytes with tyrphostin B42 elevates the cGMP accumulation above basal and β-adrenergically-stimulated levels in pinealocytes ($p<.05$).

The results obtained for the increases in adrenergically-activated cAMP and cGMP accumulation following treatment with the tyrosine kinase inhibitor, tyrphostin B42, support previously published results of similar action for other tyrosine kinase inhibitors (Ho *et al.*, 1995; Ogiwara *et al.*, 1995). Intuitively, in combination with these previous publications, the results suggest that the intracellular accumulation of cyclic nucleotides in pinealocytes is modulated by tyrosine kinase activity under both basal states and adrenergically-stimulated conditions.

III.3. Dose-Dependent Relationship of cAMP Accumulation in Response to Tyrphostin B42 Treatment

a. Tyrphostin Alone

An examination of the pinealocyte response in terms of the measured cAMP accumulation when exposed to a range of tyrphostin B42 concentrations revealed a dose-dependent association (Fig.III.3). This study found that the cAMP accumulation was increased in a modest, though significant ($p<.05$), fashion when the

concentration of tyrphostin B42 was varied from 0.2 μM to 300 μM. Additionally, the cAMP increase was consistent ($p<.05$), though modest through all concentrations tested. This result becomes especially interesting when compared to the dose-dependent cGMP response, discussed later in this section.

b. Dose-Dependent Nature of Isoproterenol-Stimulated cAMP Accumulation to Tyrphostin B42

The potentiation in the β-adrenergically stimulated cAMP accumulation induced through tyrphostin B42 treatment was also observed to behave in a dose-dependent fashion (Fig.III.3). Furthermore, the increases in cAMP content in β-adrenergically stimulated pinealocytes were much larger in magnitude than the modest increases observed in cAMP in the tyrphostin B42 alone experiments, within the concentration range investigated. Therefore, the modulation of both the basal and adrenergically-stimulated cAMP accumulation in pinealocyte can both be hypothesized to be associated with the relative actions of tyrosine kinase, such that treatment of pinealocytes with tyrphostin B42 results in dose-dependent potentiations. Although the kinetics of the effect of tyrphostin B42 alone and β-adrenergically-stimulated cAMP are different, both accumulations are potentially modifiable by the actions of a tyrosine kinase inhibitor.

III.4. Dose-Dependent Relationship of cGMP Accumulation to Tyrphostin B42

a. Dose-Dependent Nature of Basal cGMP Accumulation to Tyrphostin B42

This study revealed a markedly different pinealocyte response to tyrphostin B42 treatment in terms of magnitude of increase in cGMP accumulation compared to increased cAMP accumulation above the respective basal levels (Fig. III.4 and Fig. III.3 respectively). The pinealocyte cGMP was elevated dose-dependently and in an exponential manner with increasing tyrphostin B42 concentrations from 0.2 μM to 300 μM. The comparison of the less dramatic, more moderate response of pinealocytes in terms of cAMP accumulation to the response curve observed with increasing concentrations of tyrphostin B42 for cGMP suggests an intrinsically different and kinetically different pinealocyte modulation of the basal accumulations of these two cyclic nucleotides.

These results support the published suggestion that the basal turnover of cGMP in pinealocytes is inherently greater than the basal turnover of cAMP (Ho *et al.*, 1990). The effect of adding increasing concentrations of tyrphostin to pinealocytes increased the accumulations of both cyclic nucleotides above basal levels, but proportionally shows greater efficacy in increasing that of cGMP on its own. It can be assumed that this difference is representative of an inherent difference in the extent through which tyrosine kinase activity functions to maintain relatively low basal accumulations of both cyclic nucleotides.

b. Dose-Dependent Nature of Isoproterenol-Stimulated cAMP Accumulation to Tyrphostin B42

Isoproterenol/prazosin (10 μM) treatment of pinealocytes in the presence of a range of tyrphostin B42 concentrations resulted in a dose-dependent rise in cGMP content. However, when the tyrphostin B42 alone treated and β-adrenergically-stimulated cGMP curves are compared, it appears that isoproterenol treatment has simply an additive effect over the tyrphostin B42-induced cGMP accumulation (Fig. III.4). The majority of the rise in cGMP accumulation induced by tyrphostin B42 (300 μM) concentration and isoproterenol/prazosin can be attributed to the effect of the tyrphostin B42 on pinealocytes. This is a different result than that obtained for pinealocyte cAMP, which again illustrates the different and independent modulation of these two cyclic nucleotides. Therefore, although treatment of pinealocytes with the tyrosine kinase inhibitor, tyrphostin B42, increases the accumulations of both cyclic nucleotides, it has recognizably different effects upon measured nucleotide comparison. This study thus shows that the modulation of both cyclic nucleotide accumulations may involve the actions of tyrosine kinases, under both basal- and β-adrenergically-stimulated conditions, but also that cAMP and cGMP are modulated differently.

III.5. Time-Course Study of Tyrphostin B42-Induced cAMP Accumulation

The results demonstrate that the isoproterenol-induced cAMP accumulation increases rapidly within 5 min of isoproterenol treatment to 1.85±.34 pmoles/

100,000 cells (Fig. III.5). However, the isoproterenol-induced cAMP normally declines with longer incubations due to desensitization, such that after 60 min of pinealocyte treatment, the measured cAMP accumulation is 0.55±.07 pmoles/ 100,000 cells. Also of importance was that at all incubation times, the adrenergically-stimulated cAMP accumulation was greater in the presence of tyrphostin B42 (300 μm) meaning that the effect of tyrphostin B42 was sustained over the time course. The 5 min isoproterenol treatment in the presence of tyrphostin B42 (300 μM) (pretreated for 15 min) resulted in a cAMP content of 5.02±.40 pmoles/100,000 cells. Furthermore, the potentiation of the cAMP accumulation induced by tyrphostin B42 was maintained, even at the longest incubation time, still at 2.35±.19 pmoles/100,000 cells. Therefore, the duration of the effect of tyrphostin B42 on the isoproterenol-stimulated pinealocyte cAMP concentration is maintained over a duration of 60 min.

III.6. Time-Course Study of Tyrphostin B42-Induced cGMP Accumulation

When basal cGMP was examined in regard to incubation time of pinealocytes with tyrphostin B42 (300 μM), it was demonstrated that the potentiation occurred quickly and was maintained for up to 75 min (Fig. III.6). After a 20 min incubation of the pinealocytes with tyrphostin B42 (300 μM), the measured cGMP accumulation was 2.89±.26 pmole, and after 75 min was still 2.36±.38 pmoles/ 100,000 cells. The exposure of pinealocytes to tyrphostin B42 resulted in a significant potentiation of the basal cGMP accumulation in a rapid and reasonably long-

lasting manner.

Additionally, tyrphostin B42 was shown to be as effective at increasing the cGMP accumulation over the incubation times tested in the presence or absence of isoproterenol stimulation, when all time points are considered.

III.7. Effect of Tyrphostin B42 on Noradrenaline-Stimulated cAMP Accumulation

When both α_1 and β-adrenergic receptor pathways are stimulated via treatment with norepinephrine (10 μM), there is a significant increase, up to fifty-fold, in the pinealocyte cAMP accumulation (Fig. III.7). Since the α_1 adrenergic receptor is associated with the activation of the protein kinase C pathway, it was important for this study to examine the effect of tyrphostin B42 when norepinephrine was used as the adrenergic agonist. In the presence of norepinephrine (10 μM), the cAMP content after 13 min of incubation was 24.69±3.80 pmoles/100,000 cells, and in this experiment was potentiated in cells pretreated with tyrphostin A1 (300 μM) to 29.46±3.90 pmoles/100,000 cells, and tyrphostin B42 (300 μM) to 48.12±.40 pmoles/100,000 cells ($p<.05$). The results obtained demonstrate a consistent potentiation of β-adrenergically stimulated cAMP with tyrphostin B42, but not tyrphostin A1. Therefore, treatment of adrenergically-stimulated pinealocytes results in an increase in cAMP accumulation that can be further increased by treatment with the tyrosine kinase inhibitors, tyrphostin B42 and genistein(results not shown). Thus, even with the activation of both α_1 and β-adrenergic pathways, inhibition of

tyrosine kinase activity further elevates pinealocyte cAMP accumulations.

III.8. Effect of Tyrphostin B42 on Noradrenaline-Stimulated cGMP Accumulation

Norepinephrine treatment of pinealocytes does not increase cGMP accumulation to the same magnitude as cAMP, which further illustrates differences in the regulation of these cyclic nucleotides (Fig. III.8). This study found that tyrphostin B42 further potentiated the cGMP accumulation in pinealocytes, and tyrphostin A1 did not significantly increase the measured cGMP accumulation. The tyrphostin B42 effect was consistent in repeat experiments. The pinealocyte cyclic nucleotide accumulations can be elevated by tyrphostin treatment when either adrenergic alone or α_1 and β-adrenergic pathways are stimulated.

III.9. Effect of Tyrphostin B42 on Forskolin-Stimulated cAMP Accumulation

By using less than maximal concentrations of forskolin (10 μM) to activate adenylyl cyclase and stimulating pinealocyte cAMP accumulation, it is possible to make inferences about the site of action of tyrphostin B42 (Fig. III.9). Pinealocytes incubated for 13 min with forskolin (10 μM) had a cAMP content of 0.60±.01 pmoles/100,000 cells. When pinealocytes were treated with forskolin (10 μM) and tyrphostin B42 (300 μM), the cAMP was increased significantly above that of forskolin alone, to 1.83±.06 pmoles/100,000 cells. Alternatively, tyrphostin A1 treatment did not significantly increase the forskolin-stimulated cAMP accumulation.

The tyrphostin B42 potentiation of forskolin-stimulated cAMP is suggestive of a site of action at the level of adenylyl cyclase and/or at the level of phosphodiesterase. The tyrphostin B42 would thus be further activating adenylyl cyclase to increase the amount of cAMP synthesized and/or could be inhibiting the activity of phosphodiesterase such that the decrease in cAMP metabolism results in an observed increased accumulation. This part of the study does not necessarily exclude other sites where tyrphostin B42 may be acting, but rather acts as evidence that one site of tyrphostin B42 activity would be at the sites aforementioned.

III.10. Effect of Tyrphostin B42 on Forskolin-Stimulated cGMP Accumulation

Forskolin does not activate guanylyl cyclase to the same degree as it activates adenylyl cyclase, which is apparent by the results of measured cGMP accumulation induced by forskolin (Fig. III.10). Forskolin (10 μM) only increased cGMP accumulation to .17±.02 pmoles/100,000 cells, whereas tyrphostin B42 (300 μM) itself increased the measured cGMP content up to 1.89±.16 pmoles/100,000 cells. Further, the results did show that combined treatment of pinealocytes with both forskolin (10 μM) and tyrphostin B42 (300 μM) resulted in significantly higher cGMP accumulations above either alone. The cGMP content was 2.72±.22 pmoles/ 100,000 cells in pinealocytes treated with both forskolin and tyrphostin B42. Thus, a similar inference can be drawn about the site of action of tyrphostin B42 for potentiation of cGMP accumulation as was previously made for the cAMP potentiation, that is, tyrphostin B42 likely had at least one site of action at either or

both, the level of guanylyl cyclase to increase cGMP synthesis and/or phosphodiesterase to reduce the cGMP breakdown.

III.11. Effect of Tyrphostin B42 and Maximal Inhibition of Phosphodiesterase on cAMP Accumulation

The non-specific phosphodiesterase inhibitor, IBMX, is another useful tool in studies designed to make inferences about the site of action of drugs within the cAMP and cGMP pathways. By using a high concentration of IBMX (1 mM), it is possible to maximally block the phosphodiesterase activity, resulting in cessation of the breakdown of cyclic nucleotides. Thus, if tyrphostin B42's one and only site of action was exclusively at the level of phosphodiesterase, we would expect that it would have no additional ability to potentiate cAMP accumulation above that in pinealocytes treated with IBMX. An interesting observation was made when isoproterenol (1 μM) and tyrphostin B42 (300 μM) were used in the presence or absence of IBMX. Although treatment of pinealocytes with IBMX elevates cAMP above basal and also isoproterenol-stimulated cAMP accumulation through its inhibition of phosphodiesterase activity, when IBMX was used in conjunction with isoproterenol and tyrphostin B42, an inhibition of cAMP accumulation was noticed (Fig III.11). It is thus inferred that this result is demonstrative of a second tyrphostin B42 effect, which is only apparent when the phosphodiesterases are non-specifically inhibited by IBMX (1 mM). It appears that tyrphostin B42 may have at least one inhibitory action at some site in the signal transduction pathway for the

synthesis process of cAMP. This inhibitory site might be at the receptor, G-protein and/or the adenylyl cyclase components of this signal transduction pathway. The possible explanation of this result is that isoproterenol and tyrphostin B42-induced cAMP accumulation of 9.68±1.45 pmoles/100,000 cells was reduced to 6.18±.86 pmoles/100,000 cells when the phosphodiesterases were inhibited by IBMX. This suggests that tyrphostin B42 has multiple sites of action within the cAMP transduction pathway in pinealocytes, one that results in observations of increased cAMP accumulations and one that yields decreased cAMP accumulations only noticeable when treatment is combined with β-adrenergic stimulation and maximal phosphodiesterase inhibition.

III.12. Effect of Tyrphostin B42 and Maximal Inhibition of Phosphodiesterase on cGMP Accumulation

A similar pattern of tyrphostin B42-mediated inhibition was observed for cGMP accumulation in the presence of isoproterenol and IBMX as that for cAMP accumulation. When isoproterenol (1 μM) and IBMX (1 mM) were used to treat pinealocytes, the cGMP content was established as 8.10±1.65 pmoles/100,000 cells, but when isoproterenol, IBMX and tyrphostin B42 (300 μM) were used, a significantly lower ($p<.05$) cGMP accumulation, 3.89±.27 pmoles/100,000 cells, was observed (Fig. III.12). Therefore, tyrphostin B42 likely has multiple sites of action within the cGMP pathway of pinealocytes.

III.13. Effect of Tyrphostin B42 When Forskolin and IBMX Are Used to Potentiate cAMP Accumulation

By stimulating adenylyl cyclase using forskolin (10 μM) while at the same time maximally blocking phosphodiesterase activity using IBMX (1 mM), this study was able to make more inferences about the site(s) of action of tyrphostin B42. As expected, when forskolin and IBMX are used to treat the pinealocytes, cAMP accumulation was significantly increased to a calculated value of 2.74±.31 pmoles/ 100,000 cells (Fig. III.13). If tyrphostin B42 works at the site of adenylyl cyclase and activates it, we would expect that addition of tyrphostin B42 to pinealocytes with forskolin and IBMX would result in further increases in cAMP accumulation. However, the pinealocytes treated with forskolin, IBMX and tyrphostin had a measured cAMP accumulation of 2.67±.10 pmoles/100,000 cells, which is not significantly different from the forskolin and IBMX-induced cAMP accumulation. It is thus possible to conclude that at least one probable site of action of tyrphostin B42 is an inhibition of phosphodiesterase activity, such that when IBMX is used to maximally block phosphodiesterase, no further increase in cAMP accumulation is induced by tyrphostin B42 treatment.

III.14. Effect of Tyrphostin B42 on Cholera Toxin and IBMX-Stimulated cAMP Accumulation

By using a 45 min incubation of pinealocytes with cholera toxin (10 μg/ml) to activate the G_s-protein and stimulate cAMP accumulation, it was the purpose of this

study to determine what other site of action of tyrphostin B42 resulted in the reduction of cAMP in the presence of isoproterenol and IBMX (Fig. III.14). These experiments also demonstrated an inhibitory action of tyrphostin B42 within the level of cAMP synthesis. The cAMP accumulation detected in pinealocytes treated with cholera toxin and IBMX was 3.14±.16 pmoles/100,000 cells, which was significantly higher than that in pinealocytes treated with cholera toxin, IBMX and tyrphostin B42, which was measured as 2.06±.08 pmoles/100,000 cells. These results allude to a second site of action of tyrphostin B42 at the level of G-protein and/or adrenergic receptor, and that this action is an inhibitory one that is typically masked by the more prominent inhibition of phosphodiesterase that yields elevated cAMP accumulation in the pinealocytes.

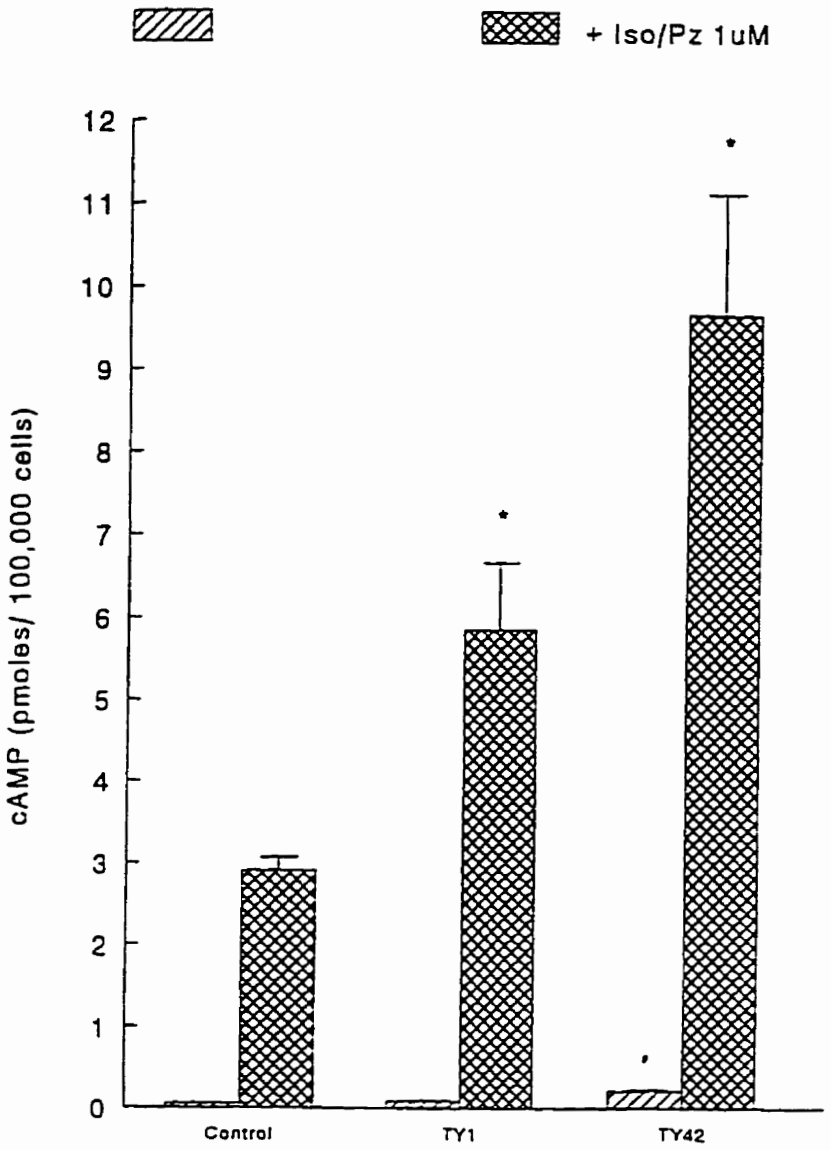


Figure III.1. Effect of tyrphostin A1 (TYA1) and tyrphostin B42 (TY42) on cAMP accumulation in pinealocytes. Both tyrphostin alone and β-adrenergically-stimulated cAMP are plotted. Pinealocytes were pretreated for 15 min with tyrphostins (300 μM) in the absence and presence of an additional 13 min of isoproterenol/prazosin (Iso/Pz) (1 μM) exposure (subsequent Iso=Iso/Pz). At the completion of the incubation, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells. *Significantly different ($p<.05$) from corresponding control. #Significantly different ($p<.05$) from corresponding control.

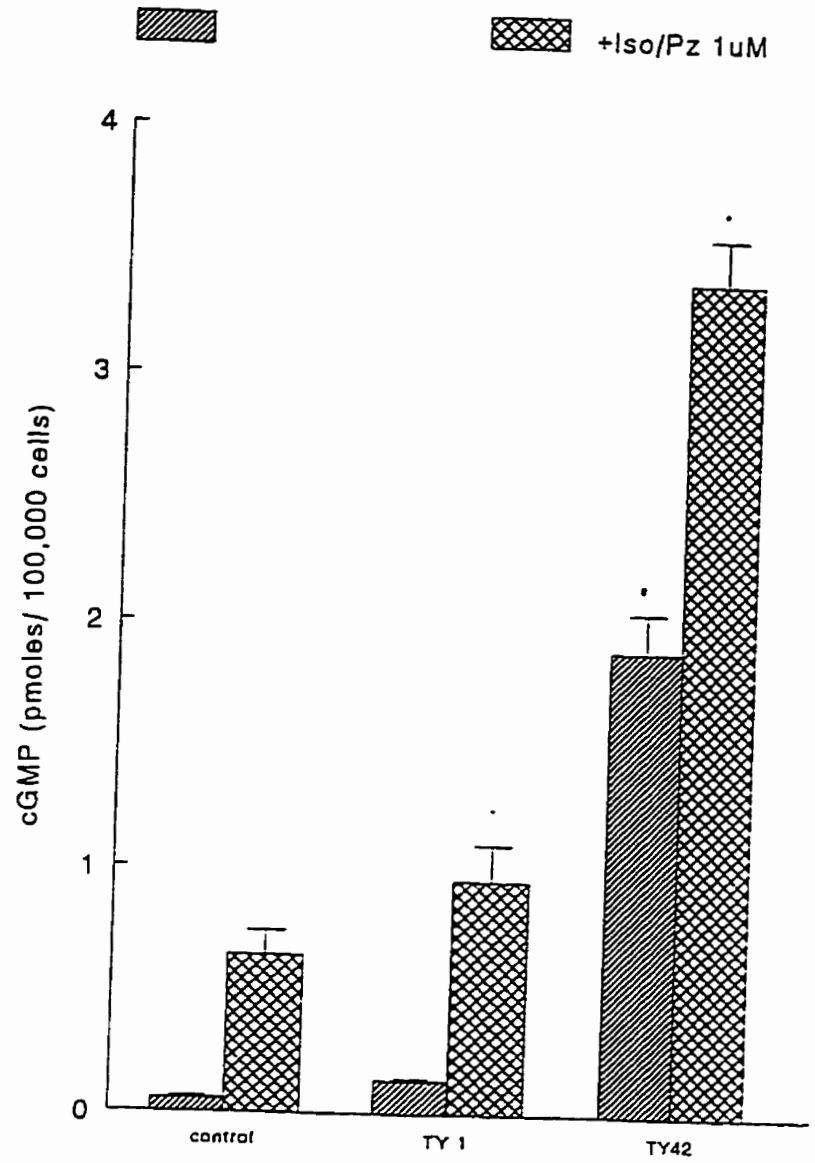


Figure III.2. Effect of tyrphostins A1 and B42 on pinealocyte cGMP accumulation. Both tyrphostin alone and β-adrenergically-stimulated cGMP are plotted. Standard 15 min tyrphostin pretreatments were utilized for adrenergically-stimulated pinealocytes. Tyrphostin A1 (TYA1) (300 μM) and tyrphostin B42 (TY42) (300 μM) were exposed for a total of 28 min. β-adrenergic stimulation was achieved by the inclusion of isoproterenol/prazosin (1 μM) for the final 13 min of incubation. At the end of the exposure time, the cellular cGMP content was determined. Each point represents the mean ± SEM of cGMP determination on four samples of cells. *Significantly different ($p<.05$) from corresponding control. #Significantly different ($p<.05$) from corresponding control.

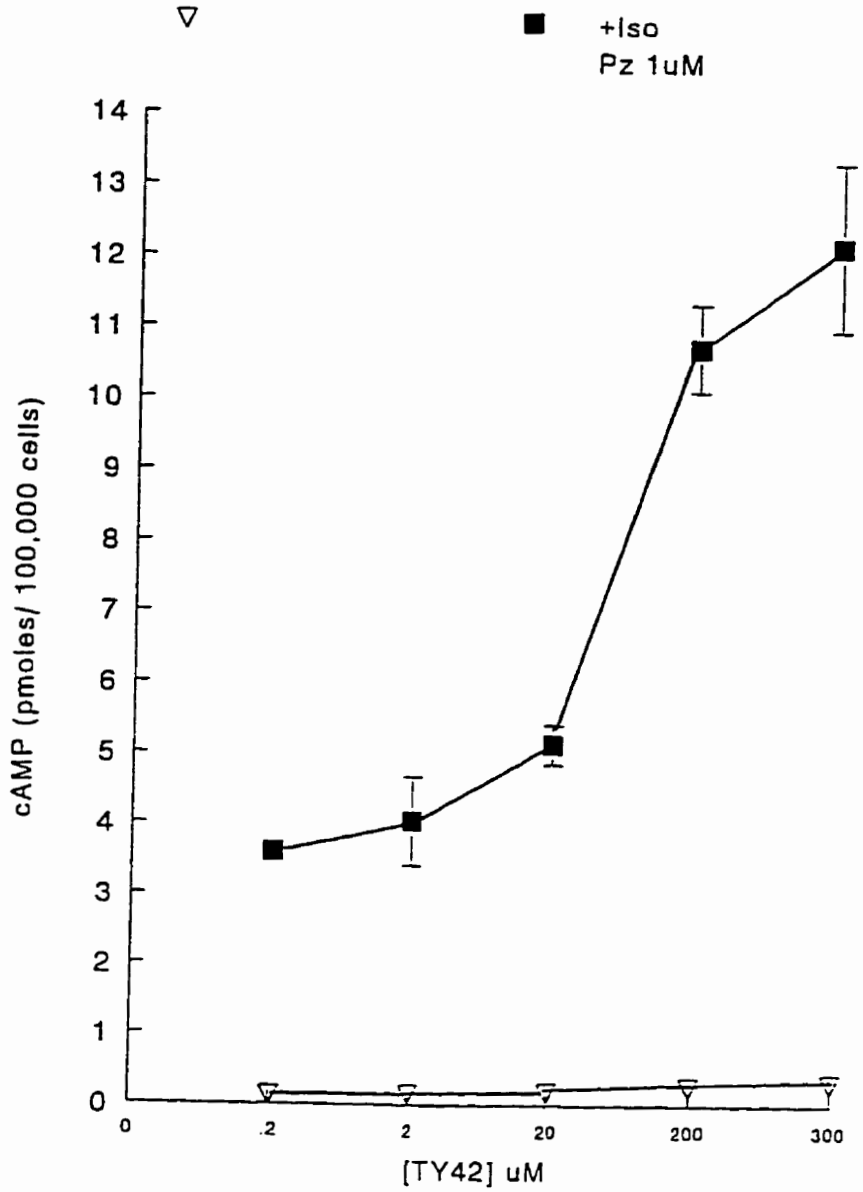


Figure III.3. The dose-dependent relationship of tyrphostin B42 and both the basal- and β-adrenergically-stimulated cAMP accumulations in rat pinealocytes. Pinealocytes were exposed to graded concentrations of tyrphostin B42, with and without isoproterenol (1 μM) stimulation, for 28 min. At the completion of the incubation, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells.

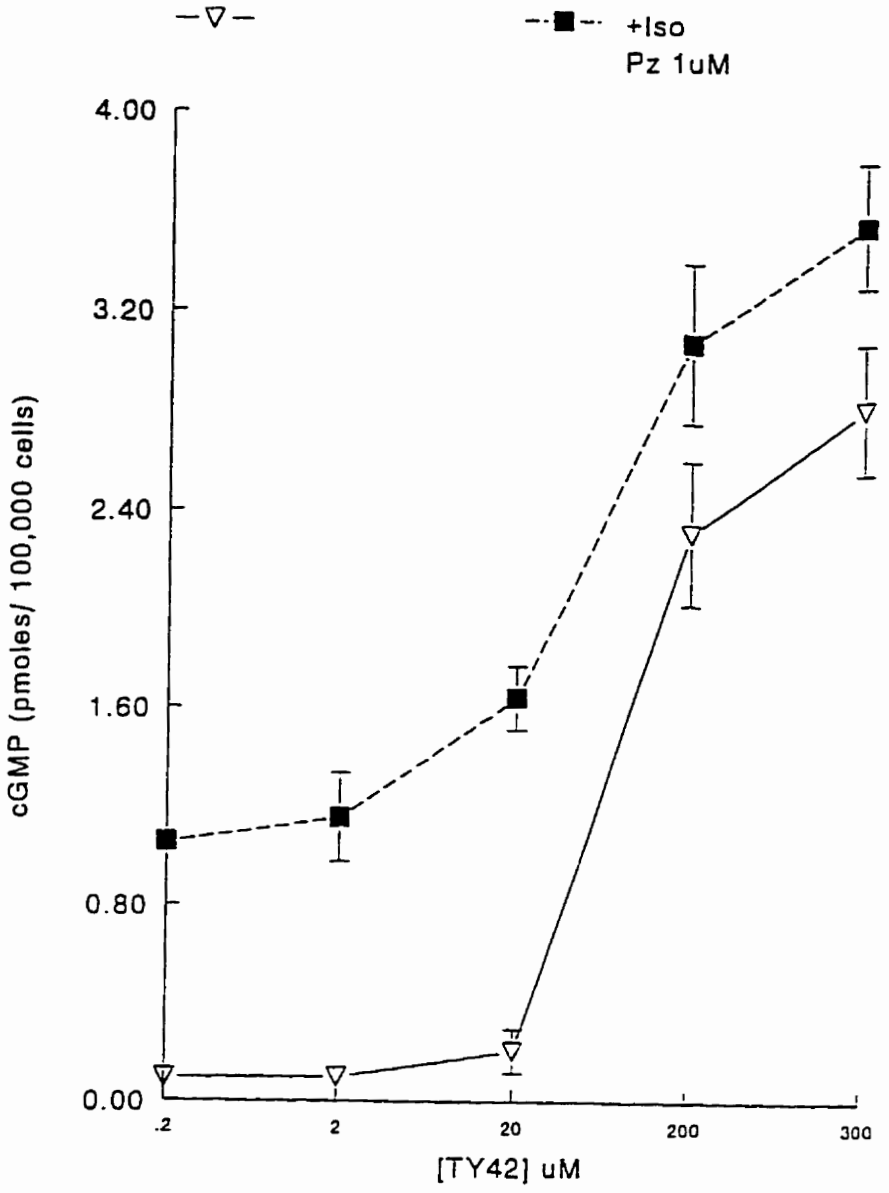


Figure III.4. The dose-dependent relationship of tyrphostin B42 and both the basal- and β-adrenergically-stimulated cGMP accumulations in rat pinealocytes. Pinealocytes were exposed to graded concentrations of tyrphostin B42, with and without isoproterenol (1 μM) stimulation, for 28 min. At the completion of the incubation, the cellular cGMP content was determined. Each point represents the mean ± SEM of cGMP determinations on four samples of cells.

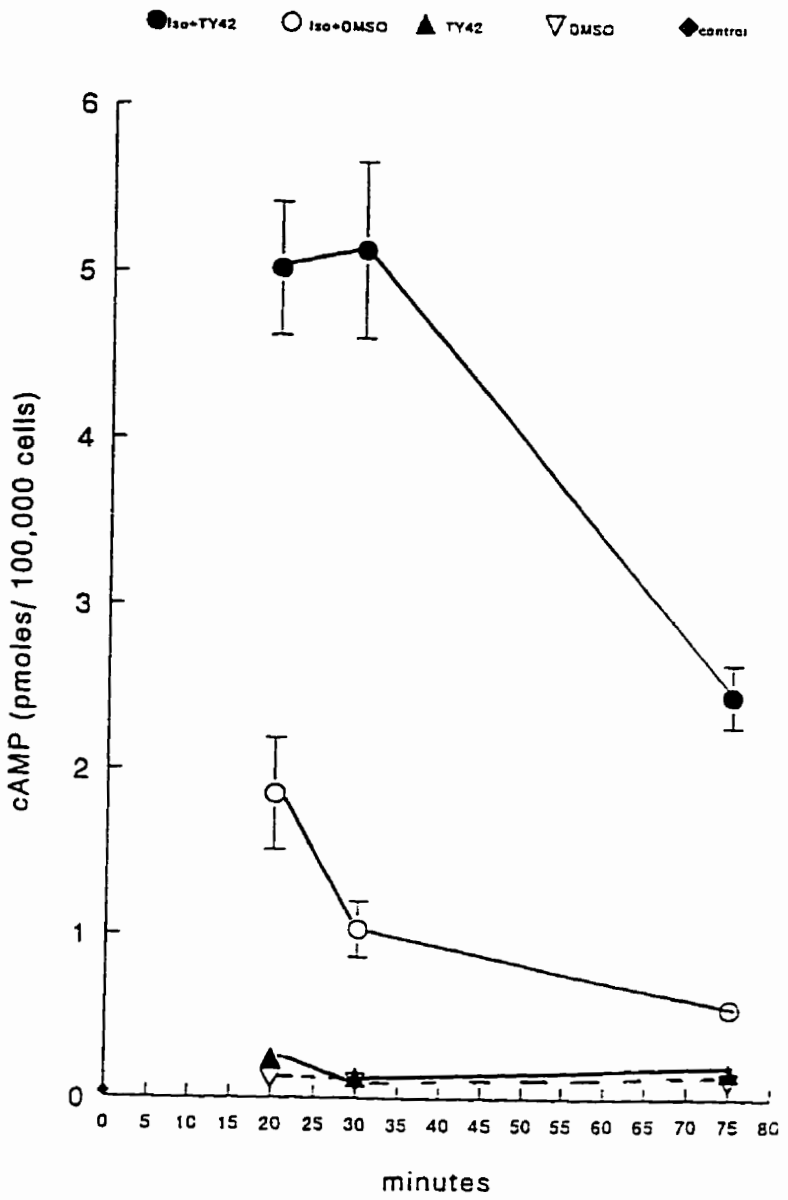


Figure III.5. The effect of tyrphostin B42 (300 μM) and duration of incubation on the observed basal- and β-adrenergically-stimulated cAMP accumulation in rat pinealocytes. The time represents the total incubation period for pinealocyte tyrphostin B42 (300 μM) exposure, which in all cases included 15 min of pretreatment of pinealocytes with tyrphostin B42. The β-adrenergic agonist was isoproterenol (1 μM). At the end of the incubation, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells.

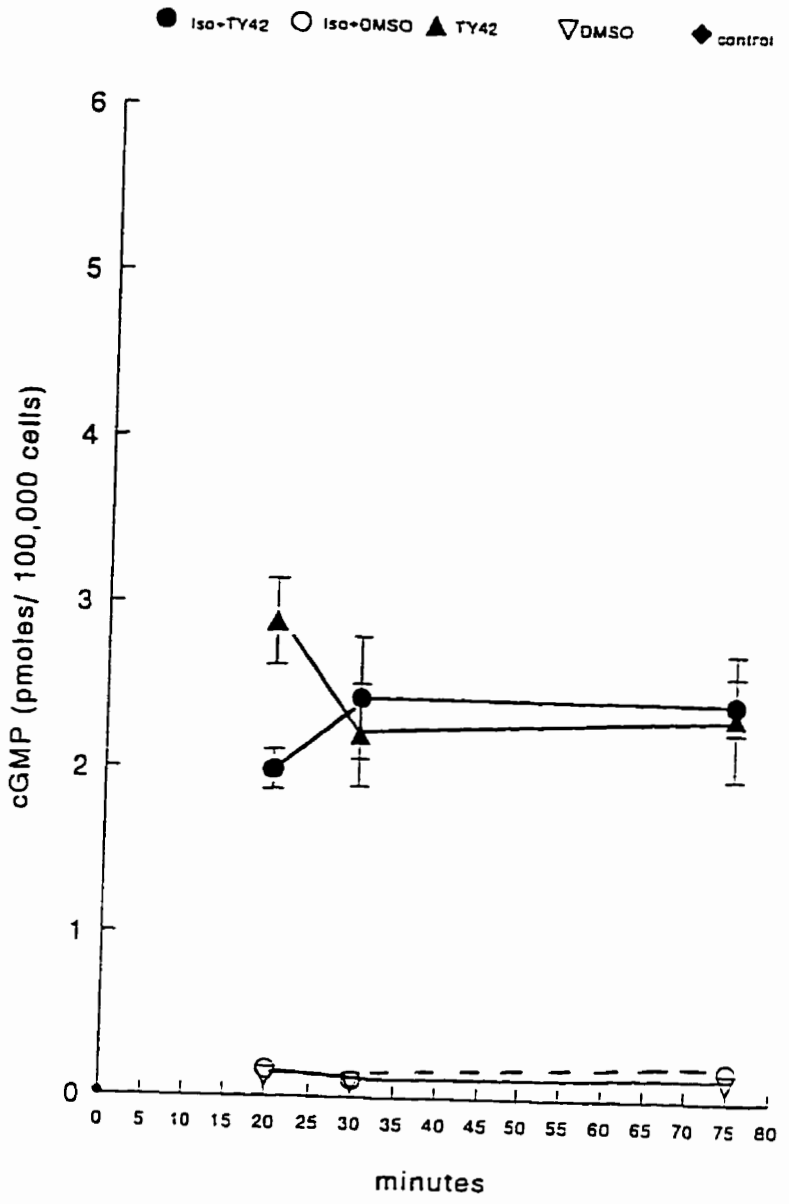


Figure III.6. The effect of tyrphostin B42 (300 μM) and duration of incubation on the observed basal- and β-adrenergically-stimulated cGMP accumulation in rat pinealocytes. The time is representative of the total tyrphostin B42 exposure time, which includes the pretreatment time of 15 min and the addition of isoproterenol (1 μM) where appropriate. At the end of the incubation, the cellular cGMP content was determined. Each point represents the mean ± SEM of cGMP determination on four samples of cells.

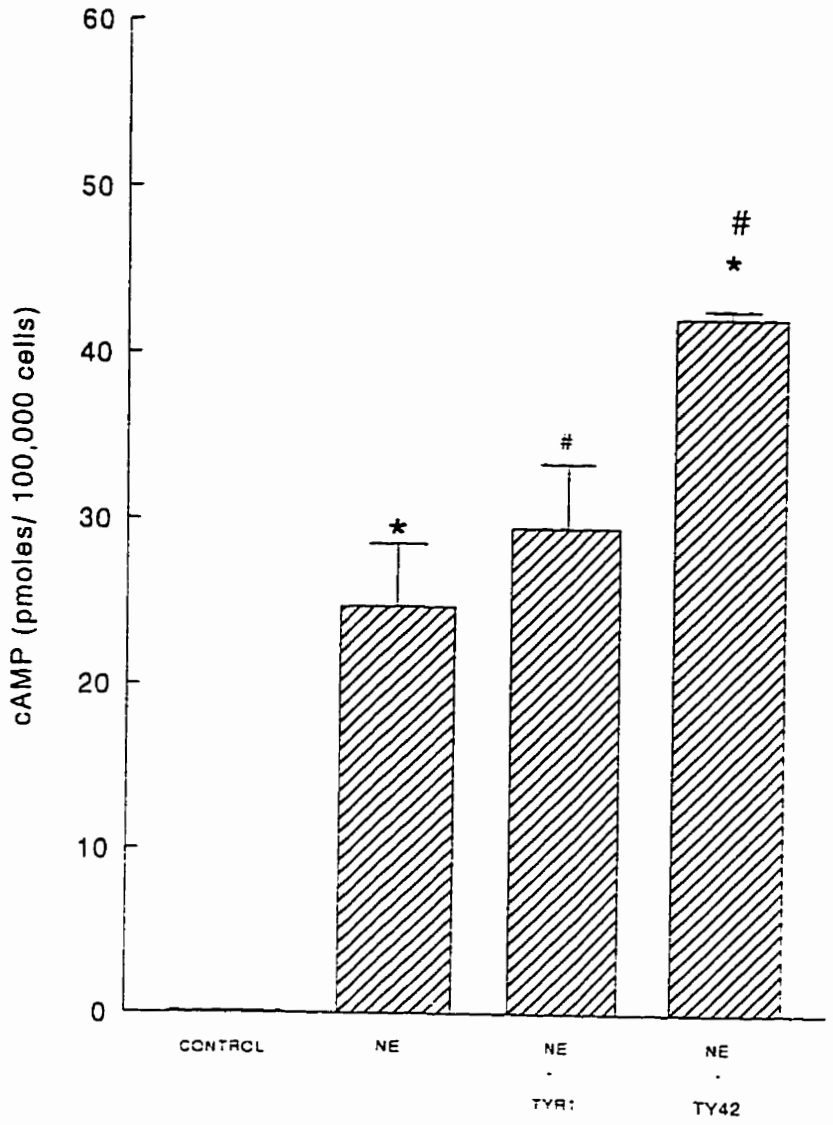


Figure III.7. The effect of tyrphostin B42 (TY42) (300 μM) and tyrphostin A1 (TYA1) (300 μM) on norepinephrine(NE) (10 μM) -stimulated cAMP accumulation in rat pinealocytes. Rat pinealocytes were pretreated with tyrphostins (300 μM) for 15 min and then administered norepinephrine (NE)(10 μM) and incubated for an additional 13 min. At the end of the incubation, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells. *Significantly different ($p<.05$) from control. #Significantly different ($p<.05$) from norepinephrine.

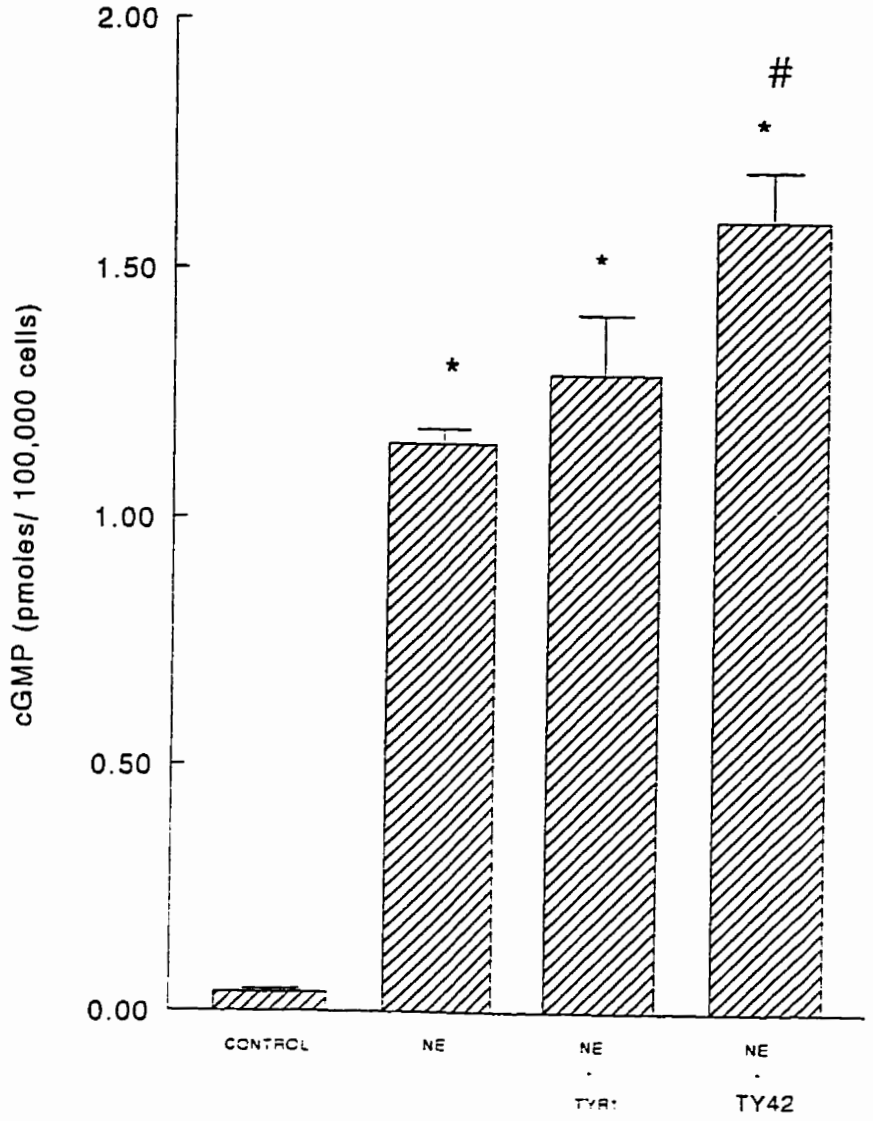


Figure III.8. Effect of tyrphostin B42 (TY42)(300 μM) and tyrphostin A1 (TYA1) (300 μM) on norepinephrine (NE) (10 μM) -stimulated cGMP accumulation in rat pinealocytes. Rat pinealocytes were pretreated with tyrphostins (300 μM) for 15 min and then administered norepinephrine (NE)(10 μM) and incubated for an additional 13 min. At the end of the incubation, the cellular cGMP content was determined. Each point represents the mean ± SEM of cGMP determinations on four samples of cells. *Significantly different ($p<.05$) from control. #Significantly different ($p<.05$) from norepinephrine.

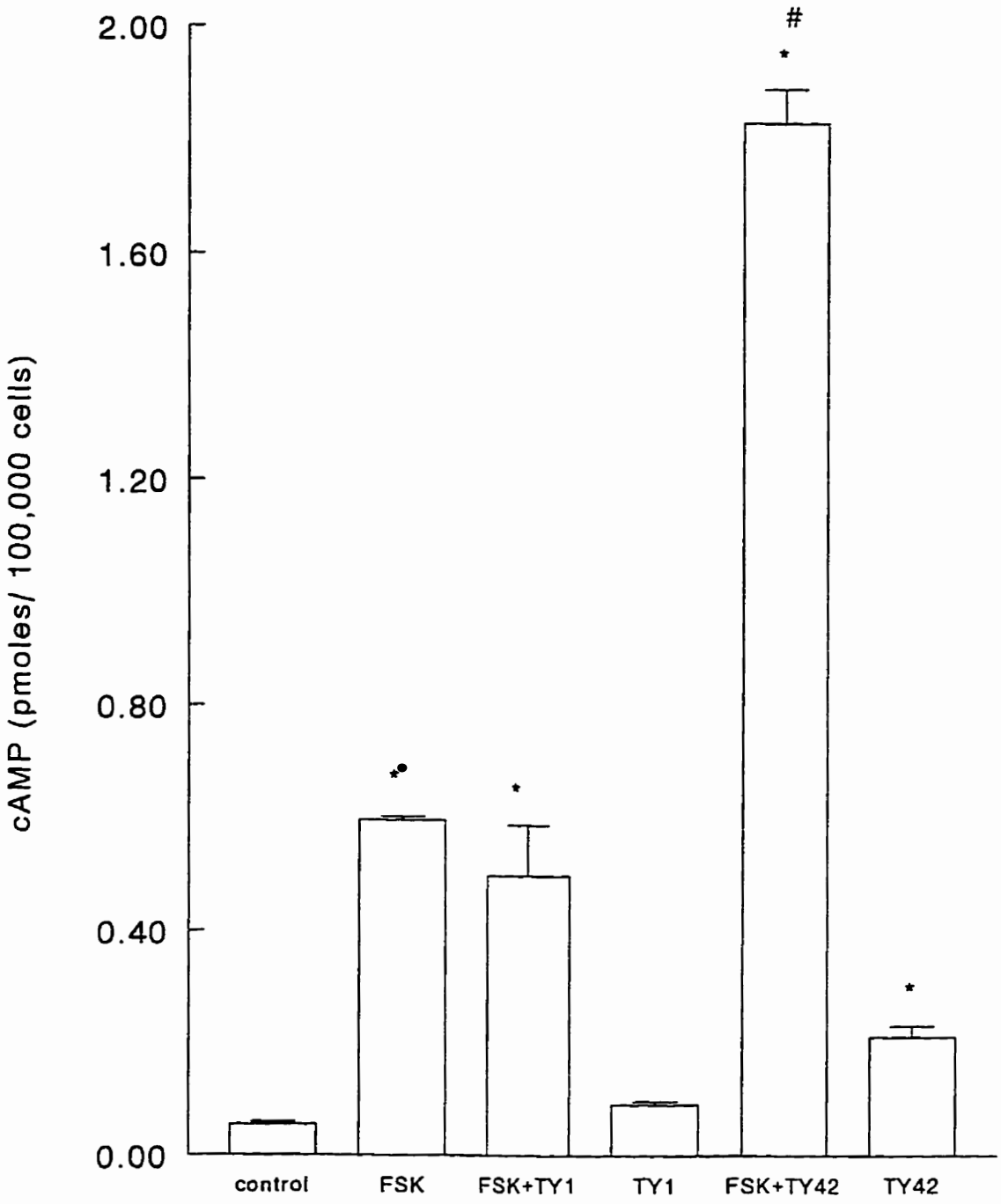


Figure III.9. The effect of tyrphostin B42 on forskolin-stimulated cAMP accumulation. Rat pinealocytes were pretreated for 15 min with tyrphostins (300 μM) where appropriate and subsequently stimulated by forskolin (FSK) (10 μM) treatment for 13 min. At the end of the incubations, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells. *Significantly different (p<.05) from control. #Significantly different (p<.05) when compared to forskolin.

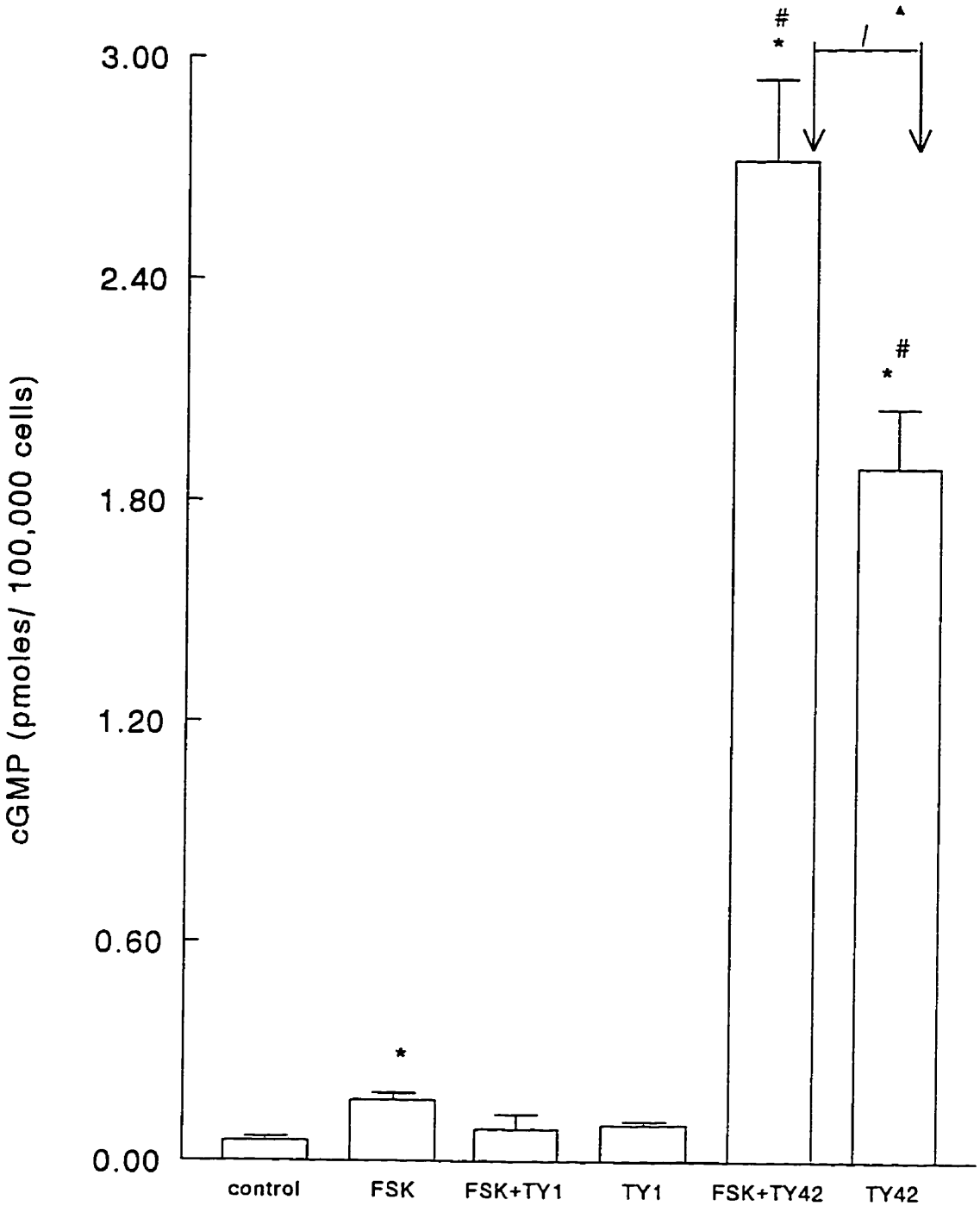


Figure III.10. The effect of tyrphostin B42 on forskolin-stimulated cGMP accumulation. Rat pinealocytes were pretreated for 15 min with tyrphostins (300 μM) and subsequently stimulated by forskolin (FSK) (10 μM) treatment for 13 min. At the end of the incubations, the cellular cGMP content was determined. Each point represents the mean ± SEM of cGMP determinations on four samples of cells. *Significantly different (p<.05) from control. #Significantly different (p<.05) when compared to forskolin. significantly different (p .05) when compared to each other.

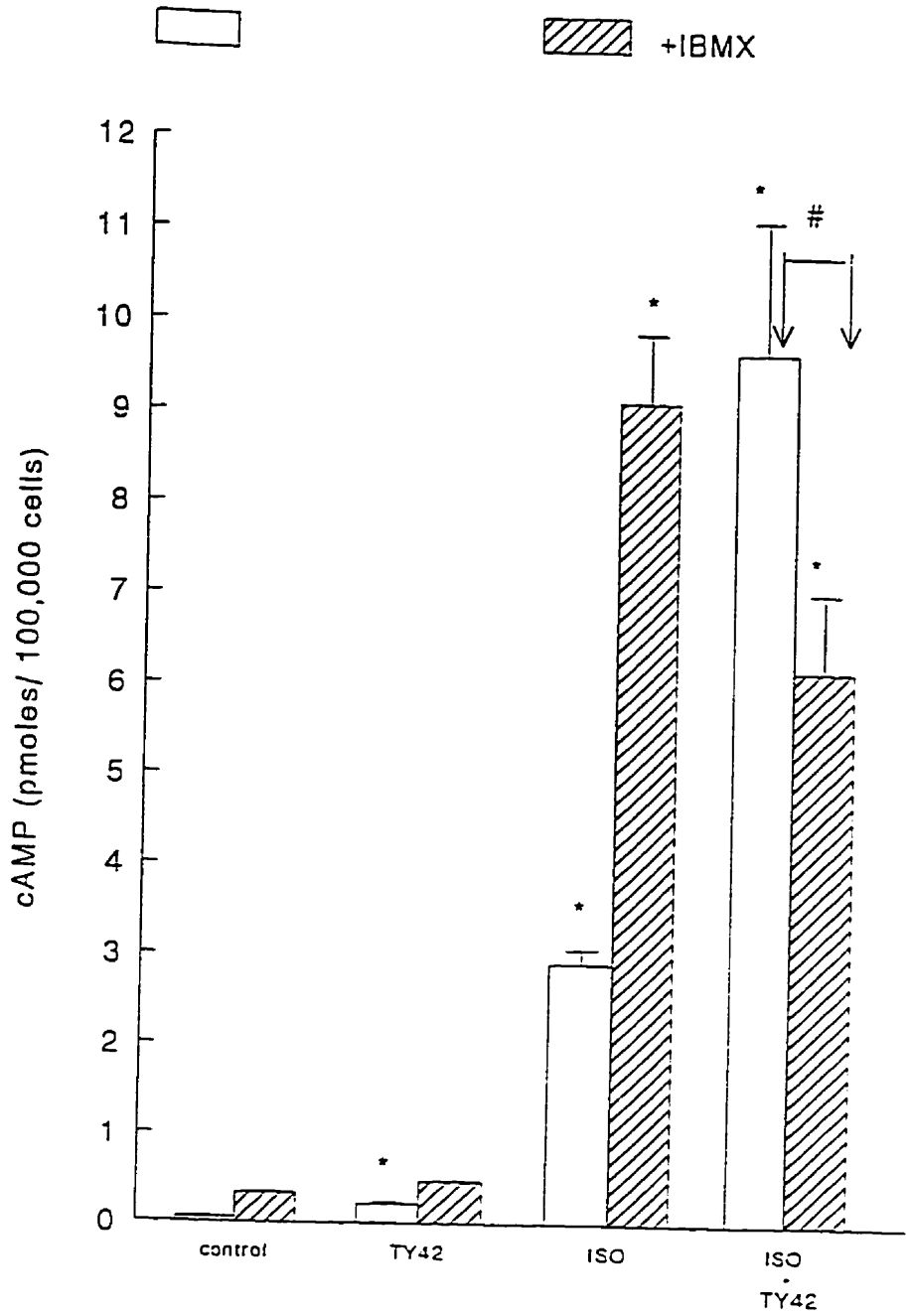


Figure III.11. The effect of tyrphostin B42 (300 µM) on isoproterenol-stimulated cAMP accumulation in the presence of maximal phosphodiesterase inhibition. Rat pinealocytes were incubated with tyrphostin B42 (300 µM) and isoproterenol (1 µM) in the presence or absence of a phosphodiesterase inhibitor, IBMX (1 mM). At the end of the incubations, the cellular content of cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells. *Significantly different (p .05) from corresponding control. #Significantly different (p< .05) from each other.

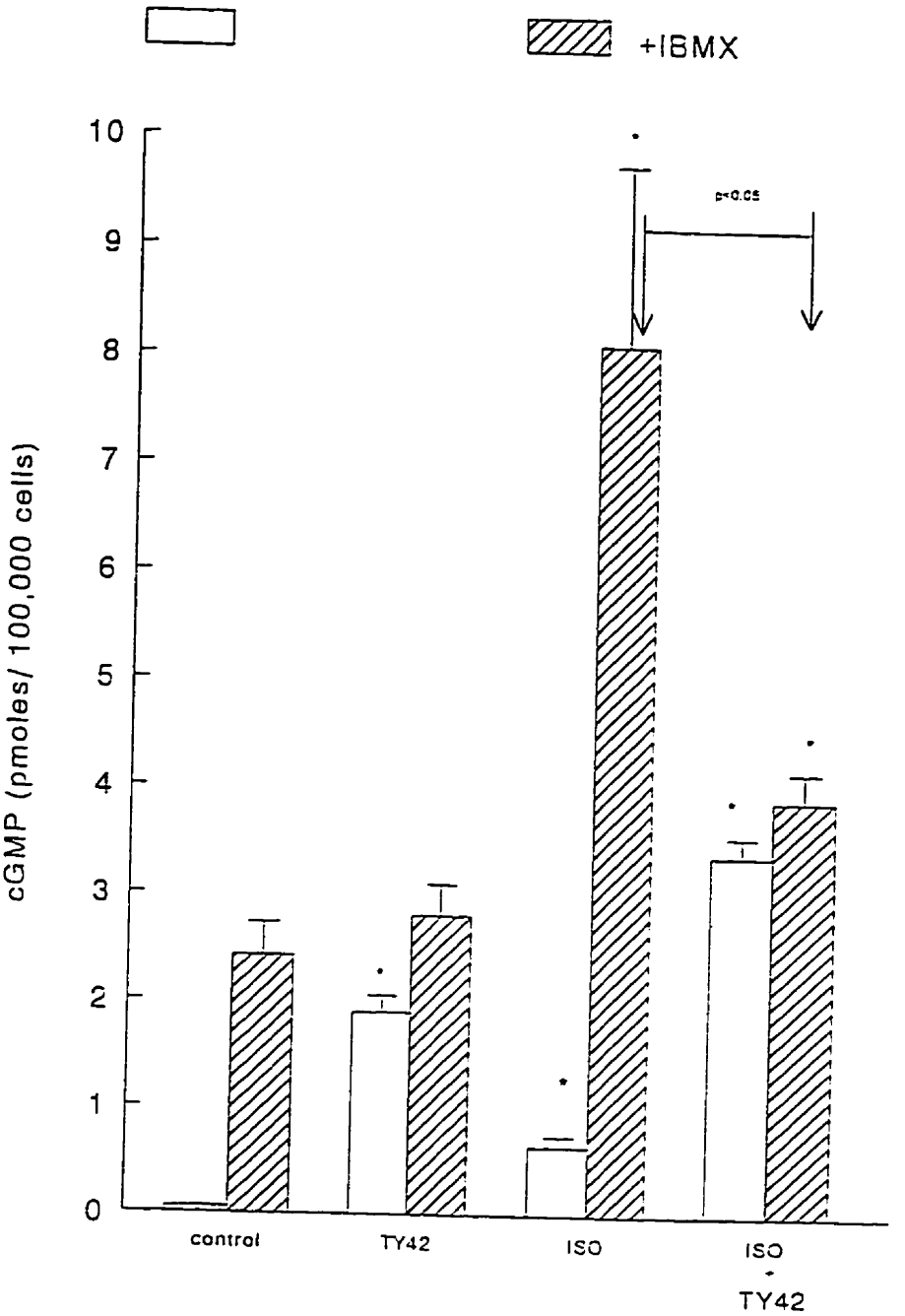


Figure III.12. The effect of tyrphostin B42 (300 μM) on isoproterenol-stimulated cGMP accumulation in the presence of maximal phosphodiesterase inhibition. Rat pinealocytes were incubated with tyrphostin B42 (300 μM) and isoproterenol (1 μM) in the presence or absence of a phosphodiesterase inhibitor, IBMX (1 mM). At the end of the incubations, the cellular content of cGMP was determined. Each point represents the mean ± SEM of cGMP determinations on four samples of cells. *Significantly different ($p<.05$) from corresponding control.

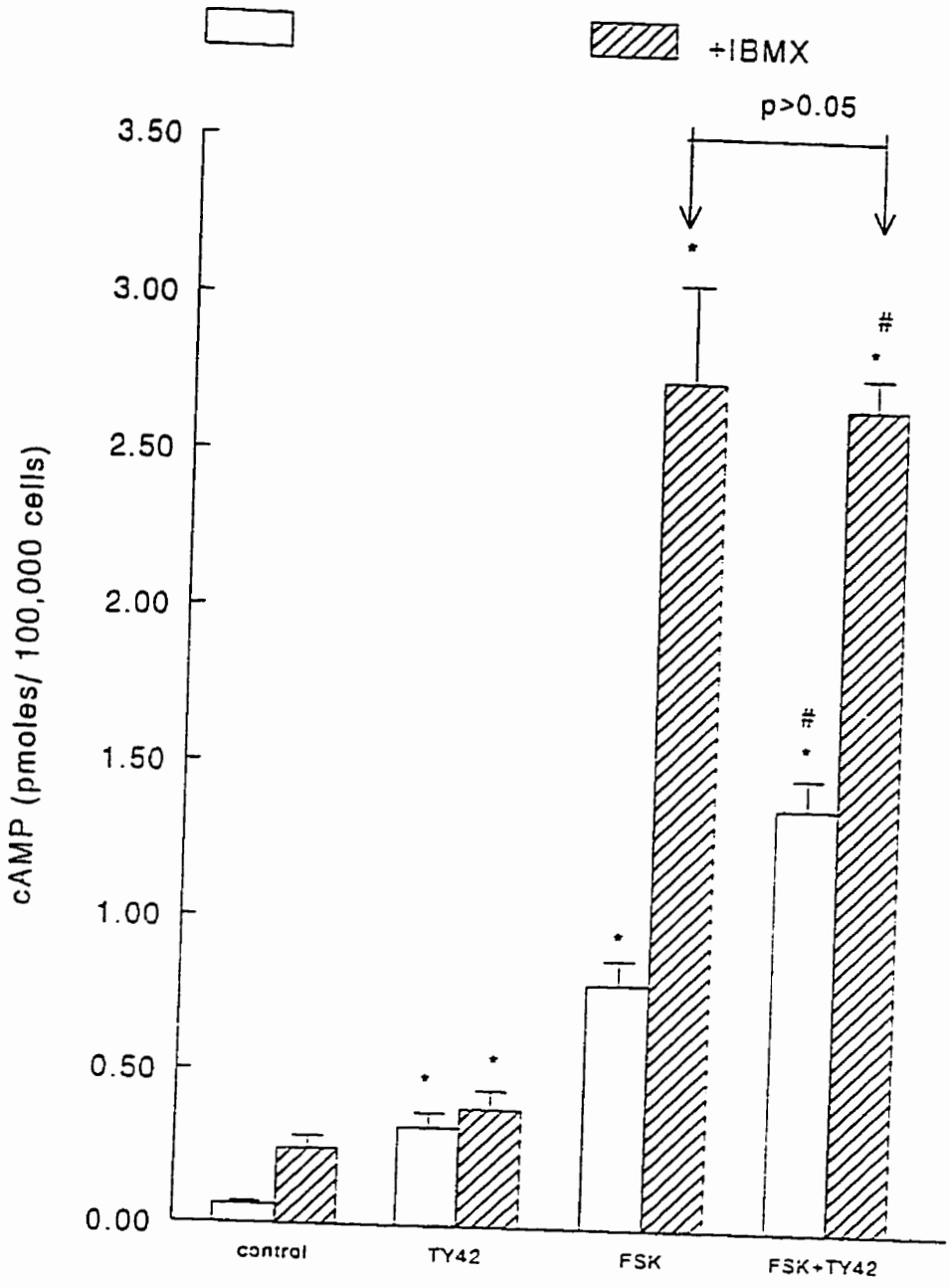


Figure III.13. Effect of tyrphostin B42 on forskolin-stimulated cAMP accumulation in the presence of maximal phosphodiesterase inhibition. Rat pinealocytes were incubated with tyrphostin B42 (300 µM) and forskolin (FSK) (10 µM) in the presence or absence of a phosphodiesterase inhibitor, IBMX (1 mM). At the end of the incubations, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells. *Significantly different ($p<.05$) from corresponding control. #Significantly different ($p<.05$) from each other.

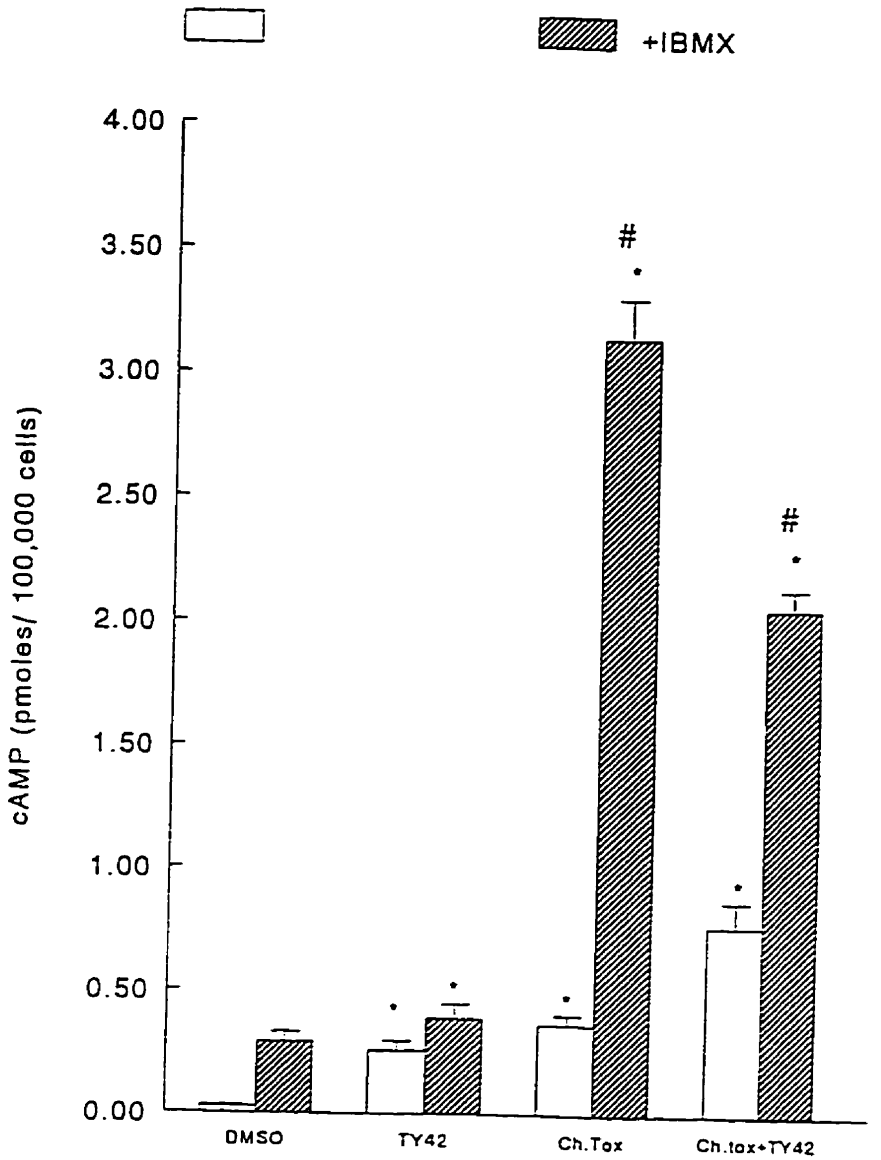


Figure III.14. Effect of tyrphostin B42 on cholera toxin-stimulated(Ch.tox) cAMP accumulation in the presence of maximal phosphodiesterase inhibition. Rat pinealocytes were pretreated for 45 min with cholera toxin(Ch.tox) (10 mg/ml), then another 15 min following addition of tyrphostin B42 (300 μM) and then additional 13 min after IBMX (1 mM) was added. At the end of the incubation, the cellular cAMP content was determined. Each point represents the mean ± SEM of cAMP determinations on four samples of cells. *Significantly different ($p<.05$) from corresponding control. #Significantly different ($p<.05$) from each other.

IV. DISCUSSION

Intracellular cAMP is perhaps the most well characterized second messenger, and more recently cGMP has also become the focus of much research indicative of their physiological relevance to signal transduction processes and cell function. In pinealocytes, the production and accumulation of intracellular cyclic nucleotides is regulated through adrenergic pathways, such that in the presence of β-adrenergic and α_1-adrenergic receptor activation, the cyclic nucleotide accumulations increase dramatically (Klein, 1985; Vanecek *et al.*, 1985). Selective stimulation of β-adrenergic receptors increases the accumulation of both cyclic nucleotides, but to a lesser magnitude than when both $\alpha_1$and β-adrenergic receptors are activated. Selective activation of α_1-adrenergic receptors results in no observed effect on the measured cyclic nucleotide accumulations (Vanecek *et al.*, 1985; Chik and Ho, 1989). Thus the activation of α_1-adrenergic receptors can not initiate increases in cyclic nucleotide accumulation on its own, but can potentiate the elevation induced by β-adrenergic activation.

Serine/threonine phosphorylation has been implicated in modulation of several levels of cyclic nucleotide accumulation, including β-adrenoceptors (Benovic *et al.*, 1985), G-proteins (Ball *et al.*, 1990) and phosphodiesterases (Manganiello *et al.*, 1995). Additionally, there are known associations between activation of serine/threonine kinases and tyrosine kinase activity, as demonstrated in β cells (Padeh *et al.*, 1991; Roifman and Wang, 1992) and oocytes (Daya-Makin *et al.*,

1991). Tyrosine phosphorylation events are critical to the transmission of growth factor messages, such as insulin, within receptive cells. Since some growth factor effects involve cyclic nucleotide pathways, as in insulin's influence on phosphodiesterase (Weler *et al.*, 1981), it seems plausible that tyrosine kinase activity within a cell might influence the cyclic nucleotide signal transduction pathways also. Indeed, it has been published that nitric oxide synthase, a major inducer of cGMP production, is altered by exposure to tyrosine kinase inhibitors in macrophages (Dong *et al.*, 1993) and in neurons (Rodriguez *et al.*, 1994).

In the first part of this study, the intent was to examine the effect of a tyrosine kinase inhibitor, that functions via direct inhibition at the substrate binding site of kinases rather than the ATP binding site, on the measured accumulation of cyclic nucleotides. The tyrosine kinase inhibitors selected from an initial screening of several different commercially available tyrphostins were tyrphostin B42, N-benzyl-3,4-dihydroxybenzylidenecyano acetamide (Levitzki and Gilon, 1991), and tyrphostin A1, 4-methoxybenzylidene malonitrile. The reason for selecting these two tyrphostins was due to their respective potencies in inhibiting EGF receptor kinase activity (as per Calbiochem published reports) as well as effecting cyclic nucleotide accumulation in pinealocytes. In my initial experiments tyrphostin B42 was the most potent at elevating pinealocyte cyclic nucleotides and tyrphostin A1 showed low potency, of the various tyrphostins that were screened. Furthermore, it has become commonplace to utilize a substance with a low relative potency as a negative control in many tyrosine kinase studies. Male rat pinealocytes were selected as the

cells to be utilized since they represent a good model of a functional endocrine cell and some initial characterization of the cyclic nucleotide signal transduction mechanisms had already been documented, not the least of which included some of my supervisor's research.

IV.1. Tyrphostins

Following exposure to 300 μM tyrphostin B42, the cAMP and cGMP accumulations were measured and compared to control pinealocytes exposed for the same time period to dimethyl sulfoxide (DMSO). It was found that tyrphostin B42 increased the cAMP accumulation, measured by radioimmunoassay, significantly above the cAMP accumulation determined for the control. Although the increase in cAMP accumulation induced by tyrphostin B42 exposure can be categorized as only moderately above the basal level, this still represents a consistent effect of this kinase inhibitor on pinealocyte cAMP. This increase in cAMP accumulation was not observed for pinealocytes treated with another tyrosine kinase inhibitor that competes with ATP, genistein (Ogiwara *et al.*, 1995). The reason for these contrasting observations may have one or more of several explanations: (1) the mechanism of inhibition of tyrosine kinases is different for genistein than it is for tyrphostin in that genistein competes with the ATP binding site (Akiyama *et al.*, 1987), therefore the specific kinases that are inhibited by these two different inhibitors may be different both in type and extent, which may include different classes of kinases for each respective inhibitor; (2) the potency and specificity of tyrosine- kinase inhibition

are quite probably different. Treatment of the pinealocytes with 300 μM tyrphostin A1, a protein tyrosine kinase inhibitor that has been characterized as a less potent inhibitor of EGF-receptor kinase activity, did not result in significant increase in cAMP accumulations which demonstrates that a less potent tyrosine kinase inhibitor can fail to elevate the cyclic nucleotide. Furthermore, if the primary difference between the structurally similar tyrphostin B42 and tyrphostin A1 is their different ranking potency to inhibit tyrosine kinase activity, we are better able to attribute the effect of tyrphostin B42 to an inhibition of a tyrosine kinase activity.

The experiments demonstrated a statistically significant increase in cGMP accumulation in the pinealocytes exposed to 300 μM tyrphostin B42. The concentration of measured cGMP accumulation in the rat pinealocytes was increased roughly 100-fold, with tyrphostin B42 treatment. An increase in cGMP accumulation has been observed in pinealocytes exposed to other tyrosine kinase inhibitors, genistein and erbstatin (Ogiwara *et al.*, 1995), but the increase in cGMP accumulation was far greater in cells exposed to tyrphostin B42.

These results suggest that the basal concentrations of cAMP and cGMP are modulated by specific tyrosine phosphorylation events such that the introduction of inhibitors to these protein tyrosine kinase(s) results in significant increases in cyclic nucleotide accumulation. The fact that the proportional increase in cGMP accumulation as compared to cAMP accumulation was far larger is indicative of a more suppressed cGMP accumulation normally induced by tyrosine phosphorylation. Furthermore, support for this effect being attributable to a phosphorylation of

tyrosine residue(s) is conferred by the observed cGMP accumulation induced by treatment with tyrosine kinase inhibitors with different structure but similar effect as tyrphostin B42. The noticeable differences in the cyclic nucleotide accumulations observed with tyrphostin B42 treatment may suggest that perhaps the basal turnover of cAMP in the pineal is lower than that of cGMP, since the measured intracellular basal accumulation of cyclic nucleotides is a function of their basal synthesis and their breakdown. There are several possible sites of action that the tyrosine phosphorylation may be affecting such that protein tyrosine kinase inhibitors change the balance in favor of larger basal cyclic nucleotide accumulations. It may be found that the cGMP accumulation may be inherently more sensitive than cAMP accumulation to phosphodiesterase inhibition if the basal levels of synthesis of cGMP are higher than that for cAMP.

IV.2. Tyrphostin B42 and β-Adrenergically-Stimulated cAMP Accumulation

In cultured pinealocytes stimulated via β-adrenoceptors using 1 μM isoproterenol/prazosin treatment, it was shown that treatment with tyrphostin B42 elevated cAMP accumulation three- to six-fold over batch matched controls. This is consistent with recently published data showing potentiation of stimulated cAMP accumulation by other tyrosine kinase inhibitors in rat pinealocytes (Ho *et al.*, 1995) and insulin-stimulated cAMP in MIN6 cells (Ohno *et al.*,1993). Treatment of pinealocytes with isoproterenol/prazosin specifically activates the β-adrenoceptor on the pineal plasma membrane, which correspondingly activates adenylyl cyclase

activity via the G-protein-coupled reaction, resulting in increased cAMP accumulation. The observed potentiation of this agonist-induced increase in cAMP accumulation by tyrphostin B42 indicates that at least one component of the cAMP synthesis pathway is inhibited to some degree via a phosphorylation event on a tyrosine residue, and/or the activity of phosphodiesterase(s) are potentiated by a tyrosine residue phosphorylation. The increase in cAMP accumulation was shown with the administration of genistein in adrenergically stimulated pinealocytes (Ho *et al.*, 1995), which supports the hypothesis that the effect of tyrphostin B42's administration is associated with inhibition of one or more protein tyrosine kinases.

IV.3. Tyrphostin B42 and β-Adrenergically-Stimulated cGMP Accumulation

These studies of β-adrenergically-stimulated cGMP accumulation demonstrated that tyrphostin B42 also increased the cGMP accumulation above that of the untreated controls. One important feature is that the increase in cGMP accumulation induced by tyrphostin B42 treatment was attributable to a greater proportion of cGMP accumulation than isoproterenol when used as a comparison. Whereas isoproterenol treatment alone increased the cGMP accumulation, tyrphostin B42 alone elevated cGMP accumulation more than twice as much. When tyrphostin B42 and isoproterenol were used in conjunction, the measured cGMP accumulation was only slightly greater than strictly additive of the two treatments alone. The significance of this observation becomes apparent when a comparison of dose-dependent cyclic nucleotide responses to tyrphostin B42 treatment is made, since

the observed effects of tyrphostin B42 are different for cAMP than cGMP. Accordingly, it can be interpreted that the differences in the cyclic nucleotide accumulations can be correlated to inherent differences in the normal modulation of the respective cyclic nucleotides. It has been previously documented that pineal cAMP and cGMP are modulated differently, as was observed with PACAP-induced cAMP accumulation, but no increase in cGMP production in pineals exposed to PACAP (Chik and Ho, 1995) and other tyrosine kinase inhibitors (Ogiwara *et al.*, 1995).

IV.4. Dose-Dependent Responses of Basal and β-Adrenergically-Stimulated Cyclic Nucleotide Accumulations

Treatment of pinealocytes with tyrphostin B42 within the concentration range of 0 to 300 μM exhibited dose-dependent increases in both cAMP accumulation and cGMP accumulation. The comparisons between the increasing tyrphostin dose effect when it is used alone and when the pinealocytes were β-adrenergically-stimulated was different in terms of the cAMP accumulation. Whereas the cAMP accumulation displayed a moderate type increase within the .2 μM to 300 μM tyrphostin B42 exposure, the agonist-stimulated cAMP accumulation was potentiated in a more pronounced fashion by increased doses of tyrphostin B42, yielding a more distinguishable change in measured accumulation with each consecutively larger dose. This is very different from the cGMP dose response curves where, in fact, both the tyrphostin alone cGMP and β-adrenergically-

stimulated cGMP curves were characterized by more substantial increases with increasing tyrphostin B42 dose. Therefore, the effect of increasing concentrations of tyrphostin B42 is functionally different for cAMP accumulation as compared to cGMP accumulation. This, of course, indicates that the effect of tyrosine phosphorylation is also very different in terms of the modulation of accumulation of each of these cyclic nucleotides. The cAMP dose response curve suggests that the effect of inhibiting tyrosine kinase activity with increasing concentrations of tyrphostin B42 only results in moderate increases in cAMP accumulation. Although similar cAMP increases were not observed in previous genistein experiments in pineal cells (Ogiwara *et al.*, 1995), genistein has been reported to increase cAMP accumulation in MIN6 cells (Ohno *et al.*, 1993). Therefore, the tyrosine kinase(s) activity that is specifically inhibited by tyrphostin B42 and is responsible for the modulation of cAMP accumulations under unstimulated conditions does not reduce the cAMP accumulations that dramatically. In contrast, when the pinealocyte is adrenergically stimulated, the tyrosine kinase(s) activity is important in modulating the magnitude of the cAMP accumulation. Thus, if we assume that increasing concentrations of tyrphostin B42 administration, more specifically inhibits the actions of some tyrosine kinase(s), then the observed dose response curve indicates that the tyrosine phosphorylation(s) effectively moderates the magnitude of cAMP accumulation. Since both the tyrphostin alone treated and agonist-stimulated cGMP dose response curves were of comparable magnitude and shape, this supports the concept that tyrosine kinase activity modulates cGMP accumulation in a much

different manner than cAMP accumulation. The pinealocyte must inherently have the propensity to synthesize cGMP, which is normally modulated by one or more tyrosine phosphorylations that act to suppress the intracellular accumulation. Hence, the administration of increasing doses of tyrphostin might inhibit the tyrosine phosphorylation that serves to maintain the basal cGMP accumulation at its typical low concentration. The comparison of the two cGMP dose response curves demonstrates that the curves are nearly parallel, indicating that the β-adrenergic stimulation appears to be additive in nature with respect to the elevation in cGMP induced through pinealocyte treatment with tyrphostin B42. This is more evidence to suggest basal cGMP synthesis and degradation rates in pinealocytes may be larger than cAMP, but balance to yield a somewhat lower basal concentration normally. These results are additionally supported by similar dose responses of pinealocytes to treatment by the protein tyrosine kinase inhibitor, genistein (Ogiwara *et al.*, 1995). Evidence that both genistein and tyrphostin B42 increase basal cGMP accumulation in a dose-dependent manner further substantiates the hypothesis that cGMP accumulation in pinealocytes is modulated through protein tyrosine kinase activity. Moreover, the effective modulation of pinealocyte cAMP by tyrosine phosphorylation is different than modulation of pinealocyte cGMP. These results support the hypothesis that both cyclic nucleotide accumulations are tonically regulated by tyrosine phosphorylation(s).

IV.5. Effect of Exposure Time of Tyrphostin B42 on Cyclic Nucleotide Accumulations

The time courses include tyrphostin treatments ranging from 0 to 75 min., under both unstimulated and adrenergically-stimulated scenarios. The study clearly demonstrates that, in spite of the decreasing efficacy of Iso/Pz-induced cAMP accumulation, in the presence of tyrphostin B42 the potentiation of the cAMP accumulation in the pinealocytes is maintained and persists over the 75 min. period. Mechanistically this suggests that the effect of the tyrosine kinase inhibitor is independent of the effect of Iso activation. Of further significance, is that when the time course of cGMP unstimulated and β-adrenergically-stimulated pinealocytes are compared, not only does the tyrphostin-induced increase persist over the 75 min trial period, but at 75 min the decreasing potentiation attributable to the adrenergic stimulation falls to such an extent that there is no statistical difference between the Isoproterenol and tyrphostin B42 and the tyrphostin B42 alone. It can be concluded that the observed potentiations of pinealocyte cyclic nucleotide accumulations induced by tyrphostin B42 are maintained for durations of at least 75 min and that the tyrosine kinase inhibitor acts independently of the Iso effect. Again, these results agree with results obtained for genistein exposures of 60 min to β-adrenergically-stimulated pinealocytes (Ho *et al.*, 1995; Ogiwara *et al.*, 1995). The observation that tyrphostin B42 enhances cyclic nucleotide accumulations in a time-dependent manner suggests that the inhibitor may be working, in part, through modulation of cyclic nucleotide metabolism.

IV.6. Effect of Tyrphostin B42 on Norepinephrine-Treated Pinealocytes

Norepinephrine is an adrenergic ligand for both the β-adrenoceptor and α_1-adrenoceptor (Strada *et al.*, 1971; Vanecek *et al.*, 1985; Chik and Ho, 1989). Treatment of pinealocytes with norepinephrine substantially elevates both cAMP and cGMP accumulation, and the activation of the α_1-adrenoceptor also results in activation of phospholipase C, and hence protein kinase C, and intracellular calcium (Sugden *et al.*, 1985; Zatz, 1985), which potentiates the cyclic nucleotide response. Genistein has been documented as potentiating, in a dose-dependent fashion, the NE-stimulated cAMP accumulation (Ho *et al.*, 1995) and NE-stimulated cGMP accumulation (Ogiwara *et al.*, 1995). The importance of NE as an adrenergic agonist for the pinealocyte is emphasized by the demonstration that, in organ culture, NE regulates melatonin production via the cAMP response element-binding protein (CREB) (Roseboom and Klein, 1995), thus *in vivo* NE is the most important regulator of pineal endocrine function. Furthermore it allowed the assessment of whether the tyrosine kinase inhibitors effect would persist even when PKC and intracellular calcium are activated. My study has shown that both cAMP and cGMP accumulations stimulated by treatment with 10 μM NE are potentiated by treatment of pinealocytes with 300 μM tyrphostin B42. This indicates that the cyclic nucleotide accumulation is modulated such that treatment with a protein kinase inhibitor increases the intracellular accumulation when the pinealocyte is stimulated with either a pure β-adrenergic agonist (Iso) or a β-α_1 adrenergic agonist. This excludes the possibility that the increase in the cyclic nucleotide accumulation upon tyrphostin

B42 treatment was solely a result of α_1-adrenergic activation and corresponding PKC stimulation and calcium mobilization. Furthermore, tyrphostin B42 potentiation of both Iso- and NE-induced cyclic nucleotide accumulation strengths the likelihood that the observed effects of the tyrosine kinase inhibitors are associated with phosphorylation events occuring within the metabolic pathways of cyclic nucleotides.

IV.7. Site(s) of Tyrphostin B42's Modulation of cAMP

The use of forskolin and cholera toxin to activate adenylyl cyclase and G-protein, respectively and to elevate cAMP accumulation in pinealocytes, was used to determine whether tyrphostin B42 acted at a post-receptor site in its modulation of cyclic nucleotides. Since tyrphostin B42 potentiated the observed cAMP accumulation in both forskolin- and cholera toxin-stimulated pinealocytes, it can be concluded that at least one of the sites of tyrphostin B42 action is at a post-receptor site. Furthermore, the forskolin data suggest that a site of tyrphostin B42 action is at the adenylyl cyclase or phosphodiesterase level. The next experimental design was to establish whether the elevation in cAMP accumulation was at the level of phosphodiesterase inhibition, by using a high dose treatment of non-specific phosphodiesterase inhibitor, IBMX.

The pinealocytes stimulated with Iso in the presence of IBMX showed cAMP accumulations above those observed for pinealocytes only treated with Iso, as would be expected. However, when tyrphostin B42 was added in combination with

Iso and IBMX, the surprising result was a reduction in the cAMP accumulation below that observed for pinealocytes treated with isoproterenol and IBMX alone. This suggests that tyrphostin B42 must also have an inhibitory effect at some site within activation of cAMP synthesis.

Through the utilization of forskolin-stimulated pinealocytes in the presence of IBMX, the addition of tyrphostin B42 did not reduce the cAMP accumulation, as was observed for isoproterenol-stimulated pinealocytes with IBMX. Therefore, the inhibitory effect of tyrphostin B42 appears to be upstream of adenylyl cyclase at the receptor or G-protein level of the signal transduction pathway. Perhaps more significantly, this result suggests that one site of tyrphostin B42 action is the inhibition of phosphodiesterase since the tyrosine kinase inhibitor did not further elevate the cAMP accumulation above the forskolin+IBMX level. Therefore the maximal inhibition of phosphodiesterase by IBMX(1mM) treatment resulted in the obscurment of the tyrphostin effect in pinealocytes activated at the level of adenylyl cyclase using forskolin. Thus phosphodiesterase inhibition by tyrphostin B42 is responsible for the increases in cyclic nucleotide accumulation observed in stimulated pinealocytes and most probably the potentiation observed in cyclic nucleotides in tyrphostin alone treated pinealocytes as well. The increase in cAMP induced by genistein in MIN6 cells was suggested to be a result of phosphodiesterase inhibition (Ohno *et al.*, 1993), as was the increases in cGMP in pineal cells (Ogiwara *et al.*, 1995).

When cholera toxin was used in the presence of IBMX, tyrphostin B42

treatment was found to inhibit the cAMP accumulation, similar to that observed for isoproterenol plus IBMX pinealocytes. These results would indicate that the second site of activity of tyrphostin B42 is most probably a small inhibition at the G-protein and/or the receptor. Since this type of inhibition has not been observed for other tyrosine kinase inhibitors, it cannot be assumed that this observation is necessarily associated with a tyrosine phosphorylation. Some support for the hypothesis that tyrosine phosphorylation is involved is gained by the results of pinealocyte treatment with tyrphostin A1. Since tyrphostin A1 represents a low potency tyrosine kinase inhibitor of similar structure , then the effects of its exposure on pinealocytes should be less than those treated with tyrphostin B42 if they are associated with tyrosine kinase inhibition.

Tyrphostin B42 appears to have multiple sites of action, such that it modulates the cyclic nucleotide accumulations at several levels. The potentiation of both tyrphostin alone treated and adrenergically-stimulated nucleotide accumulation induced by tyrphostin B42 treatment is most probably indicative of an inhibition of protein tyrosine kinase activity at the level of phosphodiesterase. This is supported by the previously cited observation that other tyrosine kinase inhibitors, genistein and erbstatin, induced a similar effect (Ho *et al.*, 1995; Ogiwara *et al.*, 1995). This study suggests that, in cultured rat pinealocytes, both cAMP and cGMP are normally modulated by the phosphorylation of one or more tyrosine residues that function to increase the intrinsic phosphodiesterase activity. When tyrosine kinase inhibitors are added, it is observed as an increase in cyclic nucleotide

accumulation manifested through an inhibition of phosphodiesterase activity.

However, it is also apparent that tyrphostin B42 has at least one other site of action which is associated with decreasing cAMP synthesis in -adrenergically-stimulated pinealocytes. This effect is usually masked by the inhibition of phosphodiesterase, but becomes apparent when phosphodiesterases are maximally inhibited using IBMX. The suspected site of this inhibition appears to be at the G-protein and/or receptor, but it has not been fully varified whether this is due to the inhibition of a phosphorylation of tyrosine residue(s) or whether it is another pharmacological action of tyrphostin not associated with its inhibitory properties against protein tyrosine kinases. These results showing the effects of tyrphostin B42 on a G-protein-mediated pathway are supported by another study on equine platelets and the inhibition of thrombin, which acts via a G-protein-coupled receptor, by tyrphostin B42 and tyrphostin B46 treatment (Dillon and Heath, 1995). This study confers supportive evidence for the involvement of tyrosine phosphorylation in cell signal transduction pathways, including those utilizing G-protein-coupled and/or cyclic nucleotide-mediated responses.

IV.8. Summary

Treatment of cultured rat pinealocytes with tyrphostin B42, a protein tyrosine kinase inhibitor, modulates basal accumulation of both cyclic nucleotides. The extent of this modulation are different for cAMP and cGMP; however, both nucleotide accumulations rise in a dose-dependent fashion to tyrphostin B42

treatment. The modulation of accumulation these cyclic nucleotides is a function of a tyrosine phosphorylation at the level of phosphodiesterase, as is supported by results of maximal inhibition of phosphodiesterase using IBMX and published results (Ogiwara *et al.*, 1995). Inhibition of the protein tyrosine kinase activity in pinealocytes through treatment with genistein and tyrphostin B42, results in inhibition of phosphodiesterase and an increase in cGMP (Ogiwara *et al.*, 1995). Similarly, the magnitude of cyclic nucleotide accumulations, especially for cAMP, following adrenergic stimulation appears to be a function of tyrosine phosphorylation as well. Tyrphostin B42 dose-dependently induces a potentiation of the cyclic nucleotide accumulations activated by β-adrenergic stimulation, and this potentiation is persistent over a period as long as 75 min. Treatment of pinealocytes with tyrphostin B42 increased the cAMP accumulation in pinealocytes stimulated by Iso, NE, FSK and Ch.tox. which are all consistent with, but not exclusive to, the tyrosine kinase inhibitor acting at the level of phosphodiesterase.

The other observed tyrphostin B42 action is an inhibition at the level of receptor and/or at the G-protein level which results in a decrease in cAMP. This effect is only observed when the cells are stimulated adrenergically or at the G-protein level with Ch.tox., in the presence of IBMX and was not observed with other tyrosine kinase inhibitors such as tyrphostin A1 or genistein. Since this effect was not observed in pinealocytes stimulated with FSK, it is unlikely that the second site of action is adenylyl cyclase, but probably upstream. It is not known whether this effect is correlated with protein tyrosine kinase inhibition, or is a result of another

chemical or pharmacological action of tyrphostin B42. A summary figure appears at the end of this section.

The increases in cGMP accumulation induced by tyrphostin B42 and genistein are due to the inhibition of phosphodiesterases (Ogiwara *et al.*, 1995), and a more recent study has established that neither tyrosine kinase inhibitor increases PKC activity (Ogiwara *et al.*, 1997). Therefore one effect of tyrphostin B42 ,a tyrosine kinase inhibitor, is the inhibition of the metabolism of cGMP and also cAMP metabolism. An extension of this finding is that one inherent function of tyrosine kinase(s) within the pinealocytes is the modulation of cyclic nucleotide accumulations. Since it is known that PKC can influence pinealocyte cyclic nucleotide accumulations (Chik and Ho, 1989), and that PKC influences receptor tyrosine kinase activities in other cell types (Cochet *et al.*, 1984; Takayama *et al.*, 1988). Perhaps the cross-talk of the PKC and cyclic nucleotide pathways are a property of the EGF and IGF receptors known to be present on the pinealocyte cell surface and/or the non-receptor tyrosine kinases within the pinealocytes.

This study further establishes that tyrosine kinase inhibitors are a useful pharmacological tool in the physiological study of signal transduction pathways in neuroendocrine tissues, and these inhibitors may play an important role in elucidating the complexities of homeostatic regulation of second messengers.

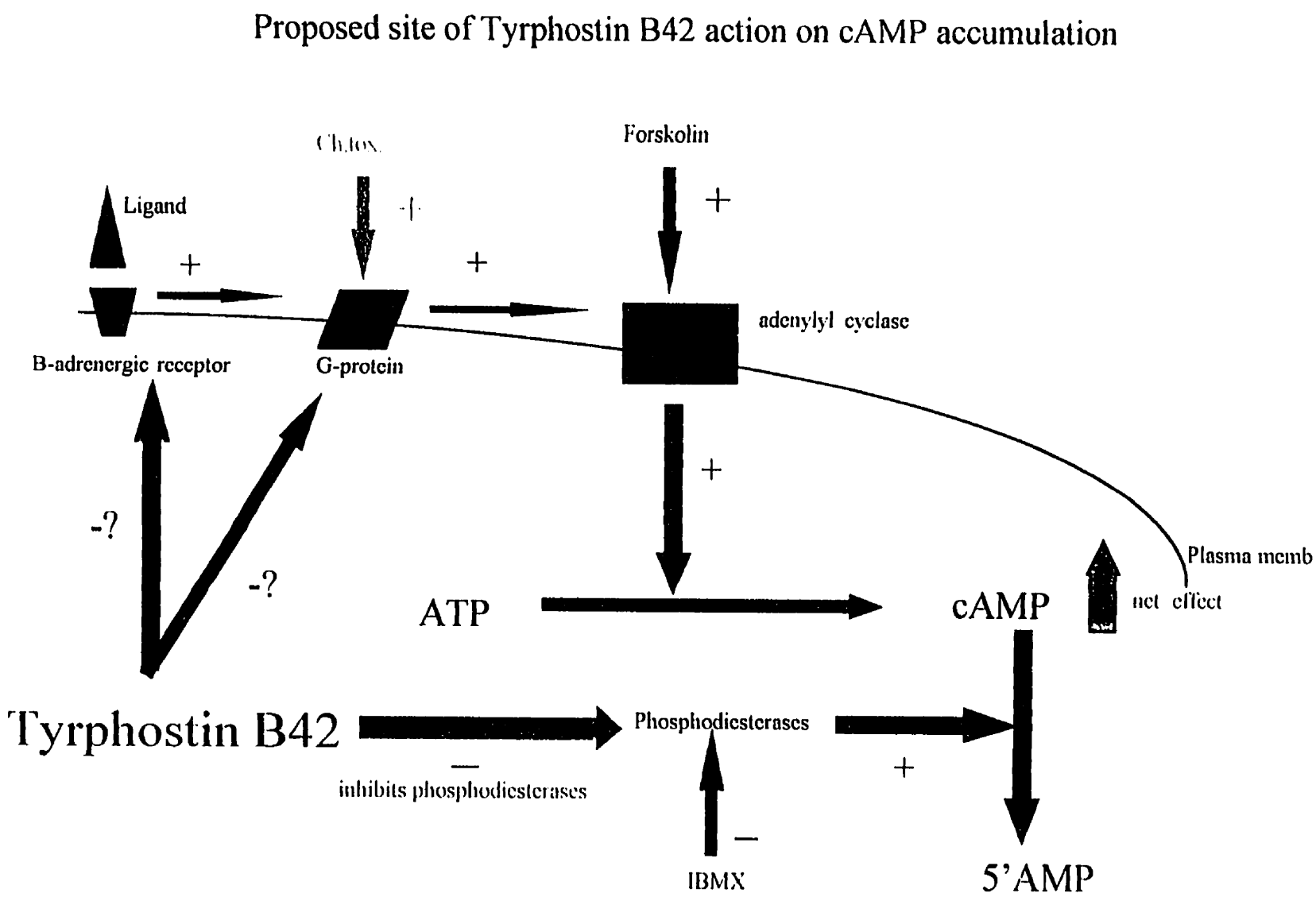


Figure IV.1. Proposed site of tyrphostin B42 action on cAMP accumulation.

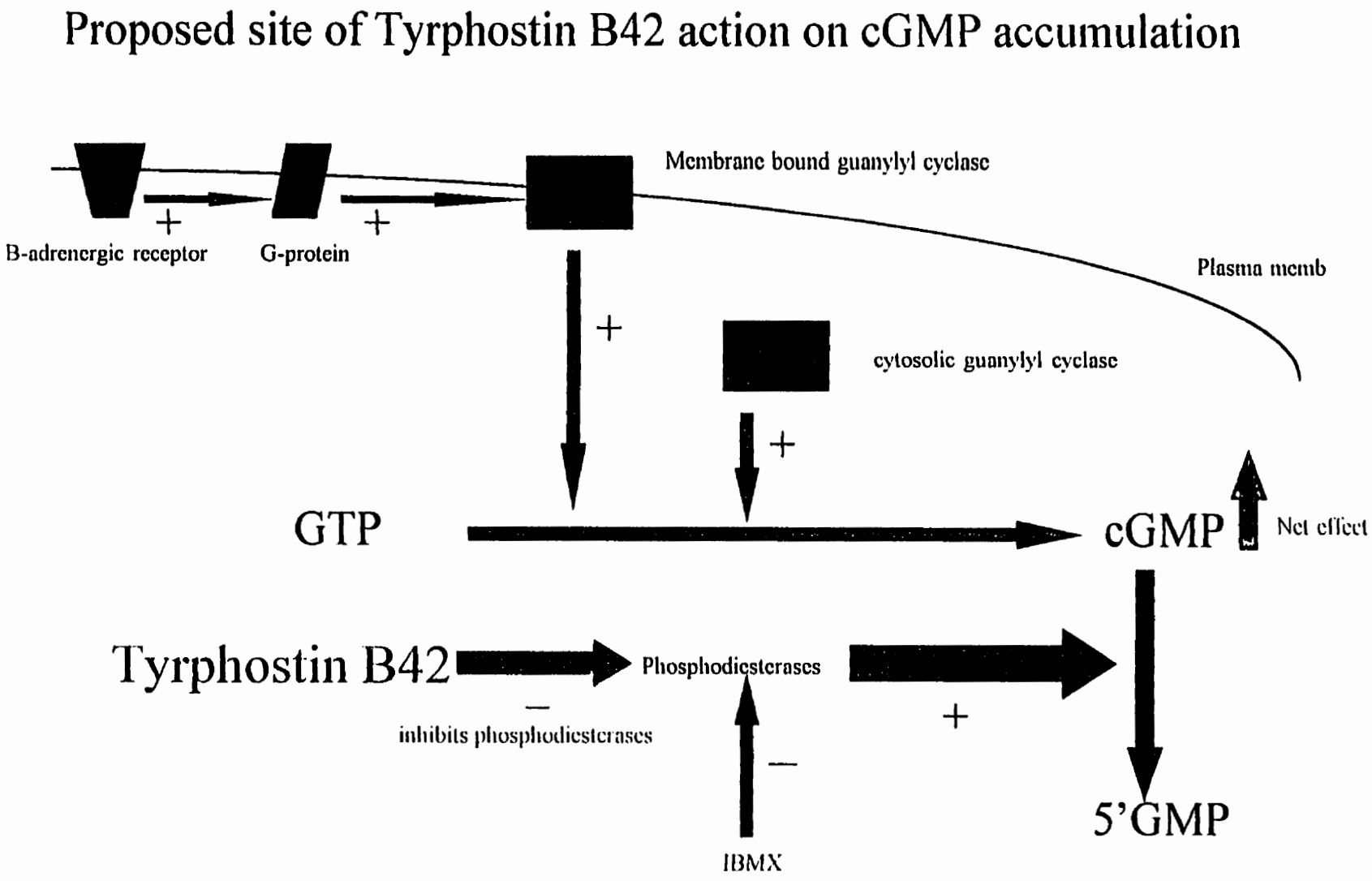


Figure IV.2. Proposed site of tyrphostin B42 action on cGMP accumulation.

V. FUTURE STUDIES

(a) Determine if the inhibitory effect of tyrphostin B42 is, in fact, at receptor and/or G-protein level, and if it is attributable to a protein kinase activity.

(b) Establish which protein tyrosine kinase(s) is inhibited by tyrphostin B42 and is responsible for the modulation of phosphodiesterase activity, and hence cyclic nucleotide accumulation.

(c) Determine whether tyrphostin B42 may have clinical uses in the treatment of diseases association with dysfunction of protein tyrosine kinase activity.

(d) Examine the effects of tyrphostin B42 in modulation of the physiological effects induced in cells by growth factors utilizing protein tyrosine kinase activity in their signal transduction process.

(e) Test other tyrosine kinase inhibitors, especially other tyrphostins, to ascertain the specific activities and potencies of these pharmacological agents.

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Appendix 1. Drug Actions

Cholera toxin:	Activates G_s protein via ADP ribosylation and inhibition of $G_{s\alpha}$-ATPase activity.
Forskolin:	Activates adenylyl cyclase, resulting in an increase in cAMP accumulation. Soluble in DMSO, stable for 4 months.
IBMX:	Broad spectrum inhibitor of phosphodiesterases. Soluble in DMSO.
Isoproterenol:	Activates both α and β adrenoceptors, but is more specific for at low concentrations. Results in an increase in cAMP and cGMP accumulation. Soluble in water.
Norepinephrine:	Equally potent agonist for and adrenoceptors. Results in increased cAMP and cGMP. Soluble in water.
Prazosin:	Antagonist of $_1$ adrenoceptors. Used in conjunction with isoproterenol to selectively activate adrenoceptors. Soluble in water with sonication.
Tyrphostins:	Inhibitors of tyrosine kinase phosphorylation activity. Result in interfering with regulatory processes which utilize tyrosine phosphorylation as a means of signal modulation. Soluble in DMSO.