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Functional expression of alpha-2 adrenergic receptor subtypes in cultured mammalian cells

Pepperl, David John, Ph.D.

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FUNCTIONAL EXPRESSION OF ALPHA-2 ADRENERGIC RECEPTOR SUBTYPES IN CULTURED MAMMALIAN CELLS

by

David John Pepperl

A Dissertation Submitted to the Faculty of the

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In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by David John Pepperl

entitled Functional Expression of Alpha-2 Adrenergic Receptor Subtypes in Cultured Mammalian Cells

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

John W. Regan	Date 6/1/94
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Josephine Lai	Date 6/1/94
Thomas Lindell	Date 6/1/94
Roger Miesfeld	Date 6/1/94

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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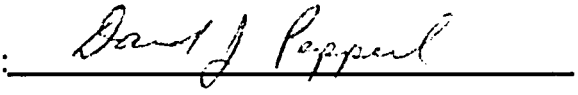
SIGNED: 

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DEDICATION

This dissertation is dedicated to my parents Dolores and Lowell Pepperl, who helped shape my past, and to my wife Stefanie, with whom I will share my future.

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ABSTRACT

The α_2 adrenergic receptors are among the most extensively studied members of the G-protein coupled receptor superfamily. They have been purified from native tissue and cloned from a number of species. Presently, three pharmacologically distinct subtypes of α_2 adrenergic receptors have been identified, termed the α_2-C10, α_2-C2 and α_2-C4. Although stable expression of these proteins in suitable host cells is commonly used for studying the pharmacology and 2nd messenger coupling of these proteins, stable expression systems are extremely time-consuming. Therefore, one focus of this work was to develop a more efficient approach for studying α_2 adrenergic receptor-2nd messenger coupling. A transient gene expression system should dramatically decrease the time required for studying receptor function.

Using a cAMP-dependent reporter plasmid and a responsive cell system, we have demonstrated transient functional expression of α_2 adrenergic receptor subtypes. Agonist activation of these receptor subtypes produces unique intracellular responses, suggesting specific receptor-effector interactions within the transfected cells. To directly address these interactions, stable cell lines expressing the adrenergic receptor subtypes were developed. Both the α_2-C4 and α_2-C10 receptor subtypes can be stably-expressed at relatively high levels in these cells. All three subtypes expressed in this cell line exhibited the pharmacology appropriate for their respective subtypes. Moreover, agonist activation of both α_2-C4 and α_2-C10 receptors in these cells produced identical dose-dependent inhibition of cAMP production.

These studies have demonstrated that α_2 adrenergic receptors can be expressed in human choriocarcinoma cells, and that agonist activation of these subtypes produces unique intracellular responses. This approach has also demonstrated the potential for regulation of gene expression by α_2 adrenergic receptors. Most importantly however, development of a more rapid functional expression system has dramatically increased our ability to study α_2 adrenergic receptor function. In the future, transient expression in JEG-3 cells should provide a useful tool for examining the effect of mutation on α_2 adrenergic receptor function. Further studies should address functional expression of other G-protein coupled receptors as well as help define the structural basis for α_2 adrenergic receptor activity.

INTRODUCTION

1.1 The Adrenergic Receptor Family

The adrenergic receptors comprise one of the largest and most extensively characterized families within the G-protein coupled receptor superfamily. This superfamily encompasses a growing array of membrane receptors, each of which exhibits a similar overall membrane topology, characterized by seven membrane spanning α-helices (O'Dowd *et al.*, 1989b). Since the 1970s, this superfamily has expanded to include muscarinic cholinergic, dopaminergic, serotonergic, prostanoid, adenosine and opiate receptors. Numerous peptide receptors including glucagon, somatostatin and vasopressin receptors, as well as other sensory receptors for taste and olfaction also belong to this growing family (Probst *et al.*, 1992).

In the most basic sense, the adrenergic receptors are the physiological sites of action for the catecholamines epinephrine and norepinephrine. Located throughout the body, the adrenergic receptors play integral roles in both the autonomic and central nervous systems. In the vasculature, adrenergic receptors control peripheral resistance, while in the heart they control both cardiac rate and contractility. In the lung, adrenergic receptors help maintain bronchial tone. Adrenergic receptors can also be found in the brain and spinal cord where they can control sympathetic neurotransmission (Ruffolo *et al.*, 1988). Since they control so many critical physiological functions, the adrenergic receptors have long been targets for numerous pharmacologic agents. β Adrenergic receptor antagonists (β-blockers) such

as propranolol are widely used in the treatment of systemic hypertension, while selective β adrenergic receptor agonists provide acute relief for bronchial asthma (Hoffman and Lefkowitz, 1990). The α adrenergic receptors are therapeutic targets as well. Both prazosin, an α_1 receptor blocker and clonidine, an α_2 adrenergic receptor agonist have successfully been used to treat high blood pressure (Ruffolo *et al.*, 1993).

The adrenergic receptors were initially classified as either α or β by Ahlquist based on pharmacological criteria. He demonstrated that at α receptors, the order of potency for agonists to evoke a physiological response was epinephrine > norepinephrine > isoproterenol, while at β receptors, this order was isoproterenol > epinephrine > norepinephrine (Ahlquist, 1948). The β adrenergic receptors were then sub-classified as either β_1 or β_2 based on agonist potency and tissue localization. Lands *et al.* examined the relative potency of sympathomimetic amines on fatty acid mobilization (lipolysis), cardiac stimulation, vasodepression and bronchodilation. They noted a marked similarity between the relative potencies for lipolysis and cardiac stimulation, and a similar relation between the potencies for inducing bronchodilation and vasodepression (Lands *et al.*, 1967). Receptors mediating lipolysis and cardiac stimulation were designated β_1, whereas those linked to bronchodilation and vasodilation were termed β_2.

Radioligand binding studies and northern blot analysis confirmed that β_1 receptors are indeed localized primarily in the heart, while β_2 receptors are concentrated in the lung and vasculature (Pepperl and Regan, 1993a). A third subtype of β receptor, termed the β_3, has only recently been identified. This receptor, localized primarily in liver, adipocytes and skeletal muscle, regulates

a number of functions including intestinal mobility, lipolysis and glycogen synthesis (Emorine *et al.*, 1989).

The β adrenergic receptors have long been targets for therapeutic intervention. β_1-Adrenergic receptors located in both the atrial and ventral myocardium can control cardiac rate and contractility. Activation of these receptors by agonists including isoproterenol can increase cardiac output, while activation of β_2 adrenergic receptors located in the vasculature promotes vessel relaxation. On the other hand, β-selective antagonists (β-blockers) such as propranolol can prevent increases in cardiac rate and contractility, thereby lowering systemic blood pressure. These agents are among the most extensively used for treatment of systemic hypertension (Hoffman and Lefkowitz, 1990). The β_2 receptors offer therapeutic targets as well. These receptors are located directly on bronchial smooth muscle, where they help control bronchial tone. Selective β_2 receptor agonists such as albuterol will promote muscle relaxation, thus providing acute relief for bronchial asthma (Ahrens and Smith, 1984).

The α adrenergic receptors, meanwhile, were first classified as either α_1 or α_2 based on their anatomical location and biochemical function. Post-synaptic α receptors located on the effector organ were termed α_1, while pre-synaptic α receptors which mediated neurotransmitter release were classified as α_2 (Langer, 1974). Similarly, these receptors were shown to mediate distinct intracellular responses; α_1 receptors coupled to inositol phosphate release, whereas α_2 adrenergic receptors mediated responses subsequent to inhibition of adenylyl cyclase (Fain and Garcia-Sainz, 1980). With development of radioligand binding techniques in the late 1970s, α

adrenergic receptors could finally be classified pharmacologically. α_1 Receptors displayed high affinity for [^{3}H]prazosin, while α_2 receptors bound [^{3}H]yohimbine much more specifically (Stark *et al.*, 1981).

With respect to α_1 receptors, studies using [^{3}H]prazosin revealed the existence of distinct subtypes of α_1 receptors in rat brain These two subtypes, termed α_{1a} and α_{1b} both possessed high affinity for [^{3}H]prazosin, but only the α_{1a} subtype also bound [^{3}H]WB4101 and phentolamine with similar high affinity (Morrow and Creese, 1986). α_1 Receptor subtypes have also been distinguished based on their sensitivity to inactivation by chlorethylclonidine (CEC). While the α_{1a} appears insensitive to this drug, the α_{1b} receptor can be totally inactivated by CEC pretreatment (Han *et al.*, 1987). More recently, three distinct subtypes of α_1 adrenergic receptors have been identified by molecular cloning. Two of these correspond to the pharmacologic α_{1a} and α_{1b} receptor subtypes, while a third subtype cloned from bovine brain has been termed the α_{1c}. (Schwinn *et al.*, 1990). Although α_1 adrenergic receptors are typically associated with smooth muscle, northern blot analysis has revealed that the α_{1a} receptor is also expressed in the hippocampus and vas deferens, while the α_{1b} receptors are expressed in the liver, kidney and spleen (Han *et al.*, 1987). Vascular α_1 adrenergic receptors are common targets in treatment of systemic hypertension. Blockade of these α_1 receptors by selective antagonists such as prazosin can decrease peripheral resistance and overall cardiac preload with little or no reflex increase in heart rate (Gerber and Nies, 1990).

1.2 α_2 Adrenergic Receptors

Following the initial classification of α adrenergic receptor into α_1 and α_2 subtypes, radioligand binding studies were pointing to the existence of multiple subtypes of α_2 receptors as well. Cheung *et al.* showed that prazosin labelled α_2 receptors in rat brain with much higher affinity compared to human platelet α_2 receptors (Cheung *et al.*, 1982). In another study, neonatal rat lung was shown to express a single α_2 receptor population with high affinity for prazosin, while human platelets expressed another with much lower affinity for prazosin. The human platelet receptor was subsequently termed the α_{2A}, while the neonatal rat lung receptor with high affinity for prazosin was designated the α_{2B} subtype (Bylund, 1985). A third pharmacologically-distinct subtype of α_2 receptor identified in opossum kidney cells (Murphy and Bylund, 1988) was termed the α_{2C} receptor (Blaxall *et al.*, 1991).

Presently, three subtypes of α_2 adrenergic receptors have been identified by molecular cloning. The α_2-C10, α_2-C2 and α_2-C4, so named for their chromosomal localizations correspond to the pharmacologically defined α_{2A}, α_{2B}, and α_{2C} subtypes respectively (Bylund *et al.*, 1992). Structurally, these receptors each possess approximately 450 amino acids and each displays the membrane-spanning α-helical structure characteristic of the G-protein coupled receptor superfamily (figure 1). These receptors exhibit rather long, extracellular amino-termini, short intracellular carboxyl termini and a long intracellular loop between the fifth and sixth transmembrane segments. (Regan *et al.*, 1988) This region has been shown through chimeric receptor mutagenesis studies to be directly involved in coupling of the receptor to G-

proteins (Kobilka *et al.*, 1988). Both the the α_2-C4 and α_2-C10 receptor subtypes contain potential sites for N-linked glycosylation on their amino termini, while the α_2-C2 and α_2-C10 receptors possess conserved cysteine residues in their carboxyl termini which are thought to be modified by fatty acid acylation (Pepperl and Regan, 1993a). Although mutation of this cysteine in the β_2 adrenergic receptor eliminated receptor palmitoylation as well as G-protein coupling (O'Dowd *et al.*, 1989a), mutation of a similar residue in the α_2-C10 receptor had little effect on receptor function (Kennedy and Limbird, 1993).

α_2 Receptors have been characterized in several different species as well. Rat, mouse and porcine homologs of the α_2-C10 subtype have been identified, while both rat and mouse versions of the α_2-C2 and α_2-C4 subtypes have been cloned (Pepperl and Regan, 1993a). α_2 Adrenergic receptors have also been identified in non-mammalian species. Receptors with an α_2 receptor pharmacology have been characterized in the skin of lower vertebrates, where they mediate pigment cell (melanophore) aggregation, leading to a color change (Berthelsen and Pettinger, 1977). Subsequently, a fish-skin α_2 receptor has been cloned from a cuckoo wrasse (*Labrus ossifagus*) genomic library (Svensson *et al.*, 1993). This fish receptor is the first non-mammalian α_2 adrenergic receptor to be cloned and sequenced.

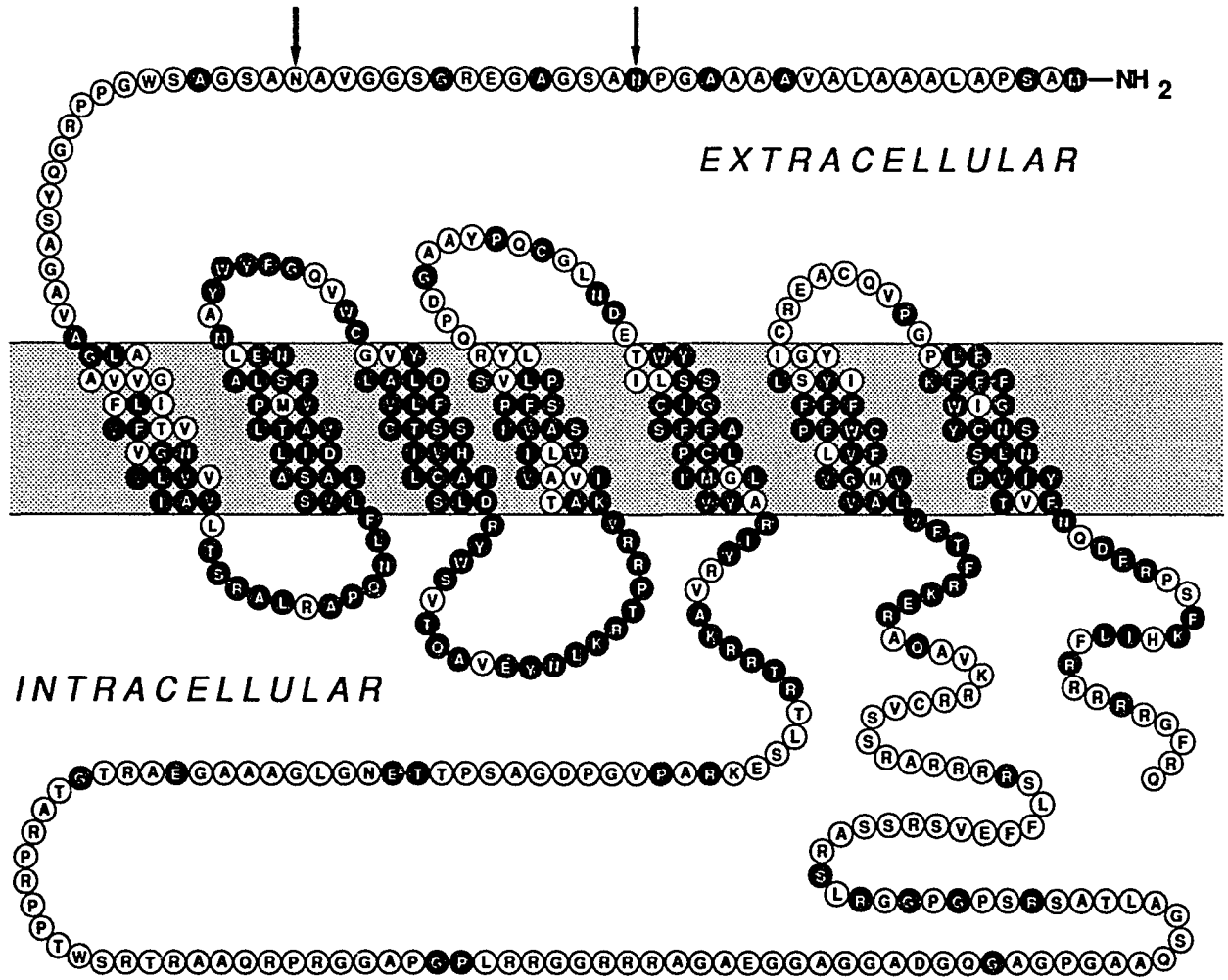


Figure 1. Structure of the Human Alpha-2 C4 Adrenergic Receptor Subtype

1.2.1 Functions mediated by α_2 adrenergic receptors

The α_2 adrenergic receptors are located throughout the body and control a wide array of crucial physiological functions. These receptors have been characterized in a number of tissues, including the central nervous system, platelets, kidney and gastrointestinal tract. In the periphery, α_2 receptors are co-localized with α_1 receptors on vascular smooth muscle, as well as on the islet β-cells of the pancreas (Ruffolo *et al.*, 1993)

The α_2 adrenergic receptors, like most other adrenergic receptors are intimately involved in cardiovascular regulation. In the central nervous system, α_2 receptors have been characterized in the brainstem, particularly in the ventrolateral medulla and locus coeruleus (Gillis *et al.*, 1985). Stimulation of these α_2 receptors decreases norepinephrine release and overall sympathetic activity, thereby lowering systemic blood pressure (Ruffolo *et al.*, 1993). This centrally-mediated inhibition of neurotransmitter release appears to account for the therapeutic usefulness of agents such as clonidine in the treatment of systemic hypertension. In a similar manner, α_2 receptor agonists have been shown to produce mild sedation and may possess therapeutic potential as an adjunct to general anesthesia (Ghignone *et al.*, 1987).

In the periphery, α_2 adrenergic receptors mediate platelet aggregation in response to epinephrine and other selected α_2-receptor agonists (Ruffolo *et al.*, 1993). Although the precise physiological role for this aggregation is not clear, it may simply represent an evolutionary response to stress or injury. Since the platelet α_2 receptor has been purified and the gene cloned (Kobilka *et al.*, 1988), this response has been attributed to the α_2-C10 receptor

subtype. In most cases however, the receptor subtypes mediating specific physiological responses are not known.

α_2 Adrenergic receptors also appear to regulate gastrointestinal secretion and motility. Activation of α_2 receptors located on autonomic fibers innervating the gut can decrease gastric acid secretion (Yamaguchi *et al.*, 1977). In fact, α_2 receptors may actually play a role in stress-induced gastric ulceration. Over-stimulation of sympathetic neurons innervating the gut has been shown to deplete norepinephrine stores. This depletion of transmitter can attenuate the α_2-receptor mediated inhibition of acid secretion, leading to gastric ulceration (Ruffolo*et al.*, 1988).

α_2 Receptors are located in the kidney, particularly in the renal cortex, proximal tubule and glomeruli (Stephenson and Summers, 1985). These α_2 receptors appear to be involved in maintaining osmotic balance, as activation of these receptors can block the vasopressin-induced reduction in sodium and water excretion in rat kidney (Smyth *et al.*, 1985).

α_2 Adrenergic receptors have also been characterized on islet β-cells of the endocrine pancreas (Fyles *et al.*, 1987). These α_2 receptors appear to be co-localized on the islets with β adrenergic receptors. Activation of β-adrenergic receptors in this tissue enhances glucose-stimulated insulin release, whereas α_2 receptor activation appears to antagonize insulin release. (Nakaki *et al.*, 1980). Subsequently, it has been shown that α_2 receptor antagonists can increase plasma insulin in the rat (Ahren *et al.*, 1984). These results imply that development of selective α_2-adrenergic agonists may hold therapeutic potential for treatment of diabetes.

1.2.2 α_2 Adrenergic receptor - effector coupling

Typically, α_2 adrenergic receptors couple to the inhibition of adenylyl cyclase via a pertussis toxin (PTX) sensitive G protein, G_i. This was first demonstrated in human platelet membranes (Jakobs *et al.*, 1976), and has since been demonstrated in stably-transfected cells expressing the various α_2 receptor subtypes (Eason, *et al.*, 1992) (Jansson *et al.*, 1994). In addition to inhibition of adenylyl cyclase, recent evidence argues for a number of distinct α_2 receptor-effector interactions. In the platelet, α_2 receptor activation also stimulates Na^+/H^+ antiport leading to increased arachidonic acid release (Sweatt *et al.*, 1985). This effect appears to involve activation of phospholipase A_2 (PLA_2) following increased Na^+/H^+ exchange, independent of any effect on adenylyl cyclase. However, in HT29 human colonic adenocarcinoma cells, which endogenously express the α_2-C10 receptor subtype, activation of PLA_2 was observed without prior Na^+/H^+ exchange (Canitello *et al.*, 1989), suggesting a direct activation of this pathway by the α_2-C10 receptor. Similar to inhibition of adenylyl cyclase, this α_2 receptor-mediated stimulation of PLA_2 could be blocked by pretreatment of the cells with PTX, suggesting involvement of an inhibitory G protein in this process (Jones *et al.*, 1991).

Depending on the cell type, the α_2-C10 may activate a variety of distinct signaling pathways. In hamster lung fibroblasts, activation of the α_2-C10 both inhibits adenylyl cyclase and stimulates inositol phosphate (IP) turnover (Cotecchia *et al.*, 1990). In stably-transfected transfected chinese hamster ovary (CHO) cells expressing the α_2-C10 subtype however, agonist elicited

an unusual bi-phasic response. Low doses of epinephrine inhibited forskolin-stimulated cAMP production, while higher doses reversed this inhibition and even potentiated cAMP levels significantly. This α_2 receptor-mediated increase in cAMP levels was insensitive to the effects of PTX, suggesting distinct effector pathways for the inhibition and potentiation of intracellular cAMP levels (Fraser *et al.*, 1989). More recently, studies using antibodies directed against the α_2-C10 receptor demonstrated that this subtype can directly interact with the stimulatory G protein G_s. (Eason *et al.*, 1992). In stably-transfected mouse mammary tumor cells however, the α_2-C10 receptor did not stimulate cAMP production, even following PTX treatment (Jannson *et al.*, 1994). It would appear then, that coupling of a specific receptor subtype to a given signal transduction pathway depends strongly on the cell type in which it is expressed.

Like the α_2-C10, the α_2-C4 receptor appears to couple primarily to inhibition of adenylyl cyclase. Cotecchia *et al.* have stably expressed the α_2-C4 in CHO cells and demonstrated that agonist activation of the α_2-C4 could inhibit forskolin-stimulated cAMP accumulation (Cotecchia *et al.*, 1990). Again, this inhibition appeared to involve a PTX-sensitive G protein, as treatment of α_2-C4 transfected cells with the toxin completely attenuated this effect. With respect to G-protein coupling, reconstitution of purified α_2-C4 receptors and G proteins in phospholipid vesicles revealed that the α_2-C4 coupled most effectively to $G_{\alpha i3}$, followed by $G_{\alpha i1}$, $G_{\alpha i2}$ and finally, $G_{\alpha o}$. This pattern was identical to that for the α_2-C10, although the extent of coupling was less for the α_2-C4. Neither the α_2-C4 nor the α_2-C10 coupled significantly to the stimulatory G protein, Gs (Kurose *et al.*, 1991). In stably-

transfected NIH-3T3 cells however, the α_2-C4 (α_{2C}) receptor coupled selectively to $G_{\alpha o}$, rather than either $G_{\alpha i2}$ or $G_{\alpha i3}$, suggesting a distinctly different pattern of effector coupling in this cell type (Coupry *et al.*, 1992)

The α_2-C4 receptor has also been shown to couple weakly to IP production as well. Stimulation of COS-7 cells expressing the α_2-C10 or α_2-C4 subtypes produced small but significant increases in IP release. This IP release was not attenuated by prior PTX treatment, implying that both subtypes can couple to independent signaling pathways involving distinct G proteins (Cotecchia *et al.*, 1990).

Although much less is known concerning the functional coupling of the α_2-C2 receptor subtype, it was recently demonstrated that the α_2-C2 receptor could inhibit adenylyl cyclase activity in membranes from stably-transfected CHO cells (Eason *et al.*, 1992) Similarly, the α_2-C2 receptor has also been shown to inhibit forskolin-stimulated cAMP accumulation in stably-transfected mouse mammary tumor cells (Jansson *et al.*, 1994). When compared to the α_2-C4 and α_2-C10 receptors expressed in this cell line, the α_2-C2 was less effective with respect to inhibition of cAMP production. Moreover, following pertussis toxin treatment, these α_2-C2 transfected cells potentiated cAMP production in response to agonist, whereas the α_2-C4 and α_2-C10 transfected cells did not. These results suggest that the α_2-C2 receptor may couple more effectively to the cAMP-stimulatory pathway than other α_2 receptor subtypes.

1.2.3 Functional expression of α_2 adrenergic receptors - Goals of this research

Clearly then, α_2 adrenergic receptors can regulate a number of important physiological functions (adenylyl cyclase activity, inositol phosphate production, etc.) by interacting with multiple effector pathways. With the cloning of the genes encoding the α_2 receptor subtypes (Lomasney *et al.*, 1990), the function of these receptors can now be examined in isolation through the use of heterologous gene expression. The ability to clone the genes encoding specific receptor subtypes and express them in an isolated cellular system has proven a valuable tool for studying both receptor function and pharmacology.

Heterologous gene expression also permits functional analysis of mutant α_2 receptors. Construction of site-directed (Wang *et al.*, 1991) and chimeric α_2 adrenergic receptors (Kobilka *et al.*, 1988) and expression of these mutants in host cell lines has helped define specific regions of the receptor required for both ligand binding and effector coupling. More recently, construction of constitutively-active mutant α_2 receptors has revealed that selected regions of the receptor, particularly those within the third intracellular loop, are required to constrain receptor activation (Ren *et al.*, 1993). Thus, heterologous expression is extremely important for our understanding of α_2 adrenergic receptor pharmacology and function. In order to facilitate the study of α_2 adrenergic receptor function and pharmacology, one of the goals of this research has been to develop a gene expression system which would permit a more rapid functional analysis of newly-cloned and mutant receptor subtypes.

Normally, in order to examine the function of a cloned receptor in an isolated system, one prepares stably-expressing cell lines which express the receptor protein. Stable expression offers the advantage that all cells in the population will express a similar amount of receptor, and hence each cell should be capable of functionally responding to agonist. The α_2 adrenergic receptors have been stably expressed in a number of cellular systems, including S115 mouse mammary tumor cells (Jansson *et al.*, 1994), NIH3T3 fibroblasts (Duzic *et al.*, 1992), CHO cells (Fraser *et al.*, 1989) and Rat-1 fibroblasts (Milligan *et al.*, 1991). Typically, cells are transfected with the gene encoding the receptor protein as well as a selectable antibiotic resistance marker, such as neomycin resistance. In a small portion of cells, these genes will integrate into the host cell genome, where they can be expressed. One can then select for antibiotic resistant cells which express the receptor protein (Kaufman, 1990a). Using this approach, Jones *et al.* demonstrated that α_2 adrenergic receptors could both inhibit adenylyl cyclase and stimulate phospholipase A_2 activity in transfected CHO cells (Jones *et al.*, 1990). One major drawback with stable expression however, is the time required to obtain a clonal population of cells. Selection of a stably-expressing population of cells can take many weeks just for a single clone. While stable expression systems clearly permit study of cloned receptor function, they are not amenable to rapid or large-scale functional analysis of newly cloned or mutant receptor subtypes.

A second, more rapid aproach is termed transient gene expression. In contrast to stable gene expression, no antibiotic selection is required, and transfected cells display a rather short-lived period (48-72 hours) of high-level

expression (Cullen, 1987). Depending on the cell type and transfection protocol, typically 0.1-20% of a cell population becomes transfected and expresses receptor protein (Kaufman, 1990b). This expression subsides with time as cells divide and lose the plasmid DNA. Quite often, COS-7 cells are used for transient studies of receptor pharmacology. The COS cells (Gluzman, 1981) are an African green monkey kidney cell line which constitutively express the SV40 large T antigen. The large T antigen is simply a trans-acting viral factor required for replication of SV40 DNA. Subsequently, plasmid DNAs containing an SV40 origin are replicated to high copy number (~10,000/cell) leading to very high levels of expression within the transfected cell population (Cullen, 1987). For these reasons, COS cells have been extensively used for large-scale production of receptor protein, such as for radioligand binding (Bylund *et al.*, 1992) and biochemical analysis (Kurose *et al.*, 1991).

Transient expression in COS cells has also been used for examining stimulatory functional responses. Following transient expression in COS cells, both α_1 and α_2 adrenergic receptors were shown to couple to stimulation of inositol phosphate turnover (Cotecchia*et al.*, 1990). Moreover, Fargin *et al.*. have expressed β adrenergic receptors in COS-7 cells, and demonstrated that these transiently-expressed β_2 adrenergic receptors can also stimulate cAMP production in response to epinephrine (Fargin *et al.*, 1989).

Unlike α_1 and β adrenergic receptors which couple to stimulatory intracellular responses (phospholipase C and adenylyl cyclase respectively), α_2 adrenergic receptors normally inhibit the activity of adenylyl cyclase. Since it is often difficult to observe inhibition of basal adenylyl cyclase levels, the

enzyme is typically stimulated first with forskolin. Forskolin is a diterpine found in the root of *Coleus forskohlii*, which directly activates adenylyl cyclase (Seamon and Daly, 1986) Addition of forskolin to cultured cells will stimulate cAMP production in the entire cell population. In a cell population transiently expressing α_2 adrenergic receptor subtypes however, only those cells taking up the DNA and expressing the receptor would be capable of inhibiting adenylyl cyclase activity. Thus it is typically difficult to observe agonist-dependent inhibition of forskolin-stimulated adenylyl cyclase activity in transiently-transfected cells. For this reason, transient expression in COS cells is not commonly used for studying the functional coupling of cloned α_2 receptors to inhibition of adenylyl cyclase.

Therefore, one of the specific goals of this research has been to develop a transient gene expression system suitable for examining the coupling of α_2 adrenergic receptor subtypes to the adenylyl cyclase signal transduction pathway. Such a system should possess the speed and ease of transient expression, while permitting measurement of functional responses to receptor activation. A transient expression system would dramatically decrease the turnaround time required for examining the effect of mutation on receptor function thereby enhancing our ability to study both agonist pharmacology and efficacy. An efficient transient expression system thus would permit rapid, large-scale screening of mutant α_2 receptor subtypes without resorting to time-consuming stable expression. Moreover, such a system should allow functional study of other receptors coupled to the adenylyl cyclase signal transduction pathway.

CHAPTER 2

DEVELOPMENT OF A TRANSIENT GENE EXPRESSION SYSTEM FOR STUDYING α_2 ADRENERGIC RECEPTOR FUNCTION

2.1 Background - cAMP and gene expression

α_2 Adrenergic receptors exert many of their cellular effects by modulating the production of the intracellular messenger cAMP (Lomasney *et al.*, 1991). cAMP is a ubiquitous intracellular second messenger produced from cellular ATP by the membrane bound enzyme adenylyl cyclase. In almost every case, β adrenergic receptors couple through stimulatory G-proteins to increase cAMP production, while α_2 receptors normally attenuate the activity of adenylyl cyclase (Summers and McMartin, 1993). Rather than exerting its intracellular effects directly, cAMP activates a specific enzyme termed cAMP-dependent protein kinase, or protein kinase A (PKA) (Figure 2). The kinase itself is composed of two catalytic and two regulatory subunits. cAMP binds to the regulatory subunits of the kinase, thereby releasing the activated catalytic subunits to phosphorylate cellular proteins (Mellon *et al.*, 1989). Activation of protein kinase A has many cellular effects, including regulation of both metabolic enzymes (glycogen synthase, lipase) and regulatory proteins, such as cFOS (Montiminy *et al.*, 1990).

Another consequence of PKA activation is an increased expression of selected genes (Roesler *et al.*, 1988). Regulation of gene expression by cAMP allows events at the cell surface (i.e. receptor activation) to induce long-term changes in cellular function by altering expression of selected proteins.

cAMP regulates expression of numerous genes, including those for tyrosine hydroxylase, proto-oncogene c-fos, somatostatin, and even the β_2 adrenergic receptor (Montminy *et al.*, 1990), (Collins *et al.*, 1990). These genes all contain within their upstream regulatory regions a specific DNA sequence (consensus = TGACGTCA) which confers responsiveness to cAMP. These cAMP-responsive elements, or CREs, contain the binding site for a regulatory transcription factor termed cAMP-responsive element binding protein, or simply CREB. Normally, CREB is bound to the CRE as homodimers (de Groot and Sassone-Corsi, 1993). With an increase in cAMP, PKA is activated and the regulatory subunits migrate to the nucleus and where they phosphorylate CREB. CREB is then thought to undergo a conformational change which permits binding of other transcription factors and as well as RNA polymerase II, permitting gene expression (Montminy *et al.*, 1990). Thus, a gene directly downstream of a CRE will increase in expression in response to elevated levels of cAMP.

Initial studies of CREB and the CRE promoter region involved transfection of a chloramphenicol acetyl transferase (CAT) reporter plasmid into human choriocarcinoma JEG-3 cells to measure CRE-dependent transcriptional activation (Delegeane *et al.*, 1987). CAT is a bacterial enzyme whch possess no mammalian counterparts, and is commonly used as a reporter gene to measure promoter activity (Alam and Cook, 1990). Introduction of such a reporter plasmid into a recipient cell line then, should provide a convenient, indirect measure of intracellular cAMP. Our approach was to transiently co-transfect cultured cells with this cAMP-responsive reporter gene and plasmid DNA encoding specific α_2 adrenergic receptor

subtypes. Cells taking up the reporter plasmid should then respond to cAMP by expressing the CAT enzyme. Instead of measuring cAMP then, CAT activity should provide a convenient, indirect measure of cAMP. We might then measure a functional response to receptor activation. Our initial studies focused on the use of COS-7 cells as the host cell line for transient co-expression. These cells have proven extremely useful for transient studies of α_2 adrenergic receptor pharmacology (Bylund *et al.*, 1992). Subsequent studies focused on the use of JEG-3 cells as a potential host cell line for transient expression. These cells contain a tissue-specific enhancer which boosts expression of cAMP-responsive genes, permitting study of cAMP-dependent promoters (Delegeane *et al.*, 1987).

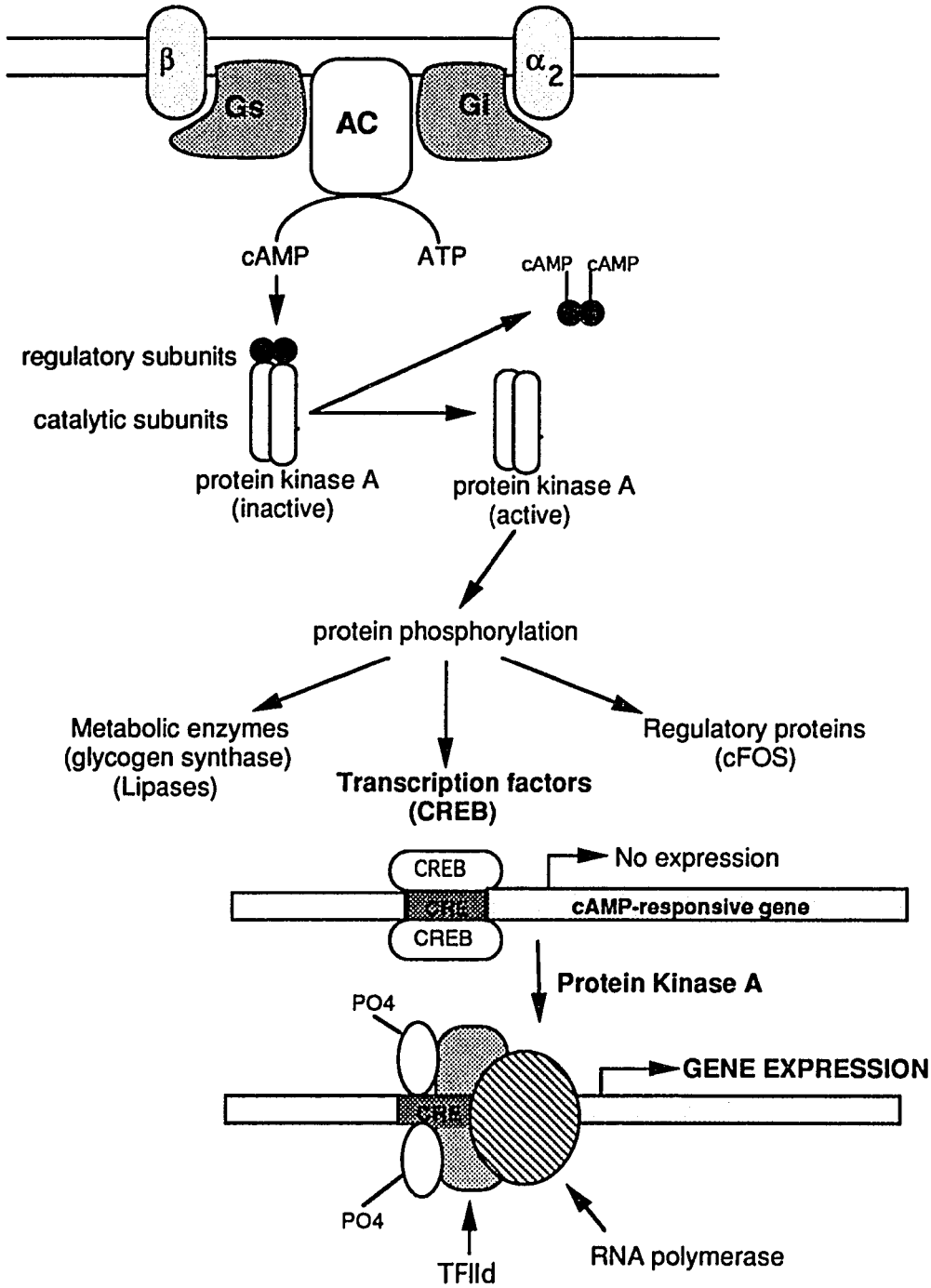


Figure 2. The cyclic AMP signal transduction cascade

2.2 Materials and methods

2.2.1 Cells and expression plasmids

COS cells (African green monkey kidney cells) were obtained from Duke university and cultured in Dulbecco's modified Eagle medium with 100 units/ml penicillin,100 μg/ml streptomycin and 5-10% fetal bovine serum (Hyclone Labs, Logan UT). Human choriocarcinoma (JEG-3) cells were obtained from American Type Culture Collection and cultured similarly in DMEM + 5-10% fetal bovine serum. The reporter plasmid used in these studies, TESBglIICRE(+)ΔNHSE, hereafter referred to as pCRE(+), was generously provided by Dr. Pamela Mellon (Salk Institute, LaJolla, CA). This plasmid contains an 18-base cAMP responsive element (CRE), from the promotor of the α-subunit gene for the human glycoprotein hormones, which has been linked to the herpes simplex virus thymidine kinase promoter, which in turn is linked to DNA encoding bacterial chloramphenicol acetyltransferase (CAT) (Delegeane *et al.*, 1987). The α_2-C2, α_2-C4, and α_2-C10 adrenergic receptor subtypes were expressed in the eukaryotic expression vector pBC12BI. This vector contains an SV40 origin of replication for high-level expression in COS-7 cells. Constitutive expression of the adrenergic receptor subtypes is driven by the RSV-LTR promoter (Cullen, 1987).

2.2.2 Reagents.

Epinephrine bitartrate, norepinephrine, oxymetazoline, propranolol, yohimbine, pertussis toxin, chloroquine, DMSO and butyryl CoA were from Sigma (St. Louis, MO). Prazosin and UK-14304 were from Pfizer, Inc. (New York, NY). Forskolin was from Hoechst-Roussel Pharmaceutics (Somerville, NJ); medetomidine from Farmos Group Ltd. (Oulu, Finland), and p-aminoclonidine from Research Biochemical, Inc. (Wayland, MA). Cell culture reagents were from GIBCO (Grand Island, NY) except for fetal bovine serum (FBS) which was from Hyclone labs (Logan, UT). [^{3}H]chloramphenicol (30-36 Ci/mmol) and [^{3}H]rauwolscine (70-80 ci/mmol) were from NEN-Dupont (Boston, MA), and mixed xylenes were from Aldrich Chemical Co. (Milwaukee, WI). DEAE-Dextran was from Pharmacia Co. (Piscataway, NJ).

2.2.3 Transfections and [^{3}H]CAT Assays.

DEAE-dextran transfections of COS-7 cells (Cullen, 1987) were performed on subconfluent cultures of cells (Appendix A). 55 cm^2 Plates were rinsed with phosphate buffered saline, and a mixture containing 0.5 mg/ml DEAE-dextran and plasmid DNA was added to the cells and incubated at 37°C. Cells were then incubated in medium containing 100 µM chloroquine. Following chloroquine incubation, the cells were exposed to media containing 10% DMSO for 2-3 min. Growth media was replaced and cells were allowed to grow for 2-3 days at 37°C.

Calcium phosphate transfections (Grahm and van der Eb, 1973) were performed on sub-confluent cultures of both COS and JEG-3 cells. Cells were plated 1-2 days before transfection. Cells were transfected with equal amounts of reporter plasmid and α_2-receptor plasmid. Briefly, cells were incubated at 37°C in DMEM/5% FBS (pH 7.1), at which time the DNA-calcium phosphate mixtures were added and incubated for 5 hours. The plates were incubated at 37°C in DMEM/10% DMSO for 2-3 min. and then maintained in DMEM/5% FBS for 36-40 hrs. Cells were rinsed with serum-free DMEM and drugs were added directly to the culture media. Immediately after the drug incubations, the cells were rinsed with PBS and scraped into Tris/EDTA/NaCl buffer. Cells were centrifuged briefly and lysed by freeze-thaw in 250 mM Tris (pH 7.5). [^{3}H]CAT assays (Seed and Sheen, 1988) were performed using 50 µL cytosol, 200 nCi [^{3}H]chloramphenicol and 300 mM butyryl CoA (Appendix B). Unless indicated, assays were for 1 hour at 37°C, and were stopped by the addition of 200 µL of mixed xylenes. Butyrylated chloramphenicol was extracted into the xylenes which were back extracted twice with 100 µL of Tris/EDTA buffer. Radiolabelled product was measured by liquid scintillation counting using a Packard Tri-Carb 460C at 50-52% efficiency.

2.3 Results

2.3.1 Transient expression in COS-7 cells

Initial attempts to develop a transient functional expression system focused on the use of COS-7 cells as the host cell line. These cells grow rapidly, are easy to maintain and can express high levels of α_2 adrenergic receptor (Regan *et al.*, 1988). Since the emphasis of this work is to develop a co-expression system, we have used a calcium phosphate transfection protocol to introduce DNA into the cells. This approach is thought to allow for much higher levels of co-transfection, compared to the DEAE-dextran technique (Keown *et al.*, 1990). Our initial studies demonstrated that the calcium phosphate protocol can be successfully used to transfect COS cells and that the ideal conditions included a 5-hour incubation with the calcium phosphate precipitates and 20 μg DNA per plate, followed by a 3 minute DMSO shock. Shorter incubations and the use of less DNA dramatically decreased α_2 adrenergic receptor expression (Data not shown).

Basal and forskolin-stimulated CAT activity were then assessed in pCRE(+)-transfected COS-7 cells following a 16-hr incubation ± 100 μM forskolin. Forskolin treatment of cells transfected with only the pCRE(+) reporter plasmid (figure 3) produced a modest increase in CAT expression (about 5-fold). Cells transfected with the α_2-C10 receptor plasmid, however exhibited a substantially increased basal activity with no observeable increase upon addition of forskolin. Since overall CAT activities were extremely low even following a 16-hour incubation with 100 μM forskolin, we proceeded to use the DEAE-dextran method to introduce plasmid DNA into these cells.

Again, COS-7 cells were transfected with either reporter plasmid alone or reporter plasmid plus α_2-C10 adrenergic receptor-coding plasmid. Cells were then incubated for 16 hours ± 100 μM forskolin. In pCRE(+)-transfected COS cells (figure 4), basal activities were extremely low, with nearly a 10-fold increase with forskolin. COS cells co-transfected with α_2-receptor coding plasmid and reporter plasmid however, displayed extremely exaggerated basal levels of CAT activity, with no substantial forskolin stimulation. Clearly, addition of the α_2-receptor plasmid dramatically increased CAT activity.

To address the source of this unusual activity, COS-7 cells were transfected using the DEAE-dextran method with either reporter plasmid alone, reporter plasmid plus pBC12BI vector or reporter plasmid DNA plus pBC12α_2-C10 plasmid. Again, cells transfected solely with reporter plasmid displayed very low activity, with a modest 4-5 fold forskolin stimulation (figure 5). As expected, cells lacking reporter plasmid exhibited no observable CAT activity (Data not shown). Co-transfection of COS cells with either reporter plasmid and pBC12BI or reporter plasmid plus pBC12α_2-C10 produced substantial increases in both basal and forskolin-stimulated CAT activity. In order to determine whether some direct chemical cleavage or modification of the chloramphenocol was occurring in the absence of CAT enzyme, CAT assays were also performed on these samples without addition of butyryl coenzyme A, a necessary co-factor for CAT enzymatic activity. These samples exhibited no detectable CAT activity (Data not shown), suggesting that this effect indeed results from actual expression of the CAT enzyme, rather than simply a chemical modification of the chloramphenicol. More importantly, these results clearly demonstrate that this effect is not dependent on the α_2

receptor or its coding sequence, as cells transfected with the vector pBC12BI also produced this effect.

In order to determine whether this large increase in basal activity was dependent on the cAMP-responsive element, we obtained a CAT reporter plasmid (TESΔNHSE) lacking a cyclic AMP responsive element from Dr. Pamela Mellon, (Salk Institute, La Jolla, CA). COS cells were transfected with either this reporter plasmid alone or reporter plasmid plus pBC12BI. Introduction of reporter plasmid plus pBC12BI into COS-7 cells again elicited a large increase in CAT expression (data not shown), suggesting that this increase in basal activity is independent of the CRE.

To summarize results with the reporter plasmid and COS cells, we have shown that α_2 adrenergic receptors can be expressed at low levels in these cells following calcium phosphate transfection. Moreover, transfection of these cells with reporter plasmid TESBglIICRE(+)ΔNHSE, elicited low levels of CAT activity which increased upon forskolin stimulation. Co-transfection of reporter plasmid pCRE(+) and pBC12BI vector DNA however, produced substantial increases in basal CAT activity. This effect appears independent of both the α_2-adrenergic receptor as well as the cAMP-responsive element, suggesting that perhaps the pBC12BI vector is directly stimulating the normally inactive CREB promoter, even in the absence of cAMP. Furthermore, both basal and forskolin-stimulated CAT activity were extremely low in COS-7 cells, even following 16 hour incubation with 100 μM forskolin. Based on the very low CAT activities observed in COS-7 cells and the unusual stimulatory effect of the pBC12 DNA, it would appear that COS-7 cells are not suitable for transient measurement of cAMP-dependent reporter gene expression.

Subsequently, JEG-3 cells, a human choriocarcinoma cell line, were used for development of a cAMP-responsive system. These cells contain a tissue specific enhancer which should boost expression of cAMP-responsive genes, providing a more robust signal compared to the COS cell system.

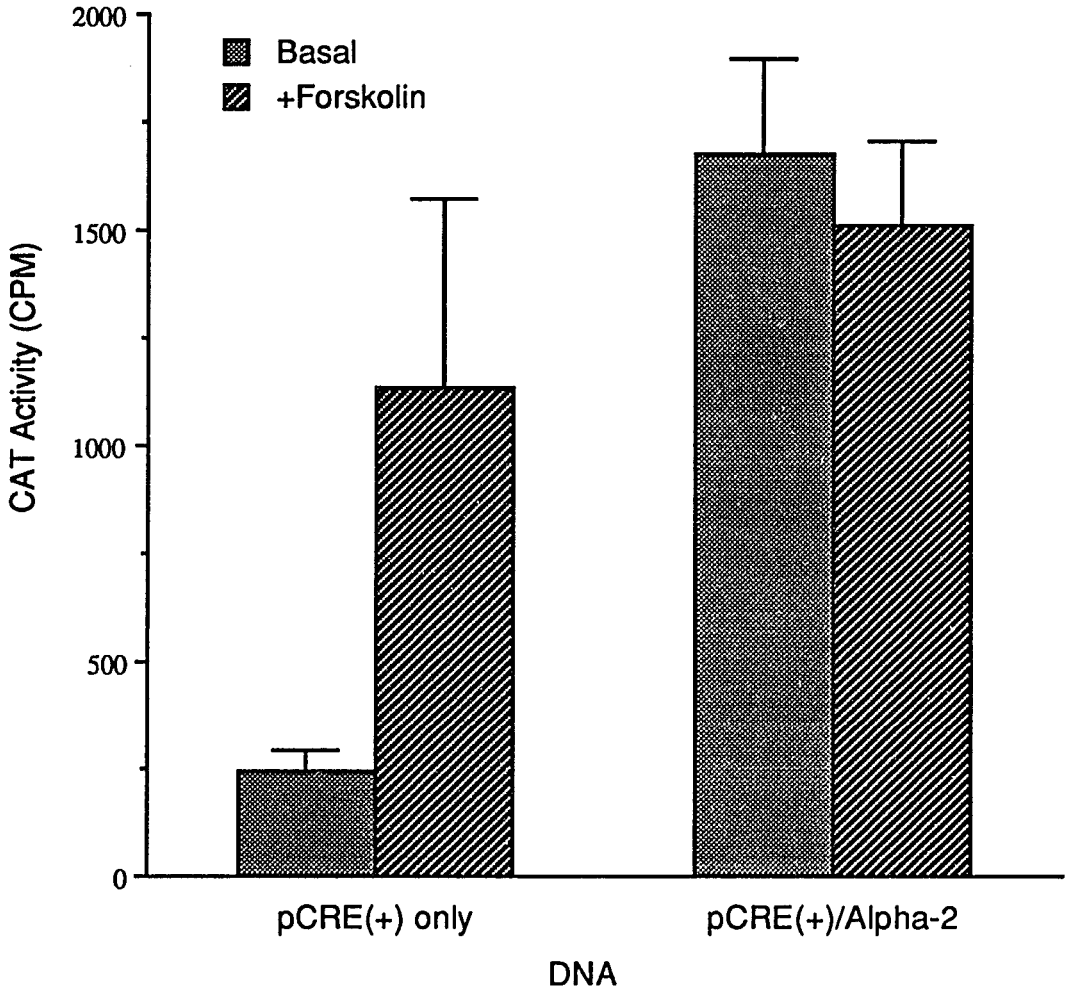


Figure 3. Basal and forskolin-stimulated CAT activity in COS-7 cells transfected using the calcium phosphate precipitation technique. Each plate received 10 μg of pCRE(+) reporter plasmid DNA and 10 μg of α_2-receptor coding plasmid. Each bar represents the mean ± S.E. of two individually-transfected plates.

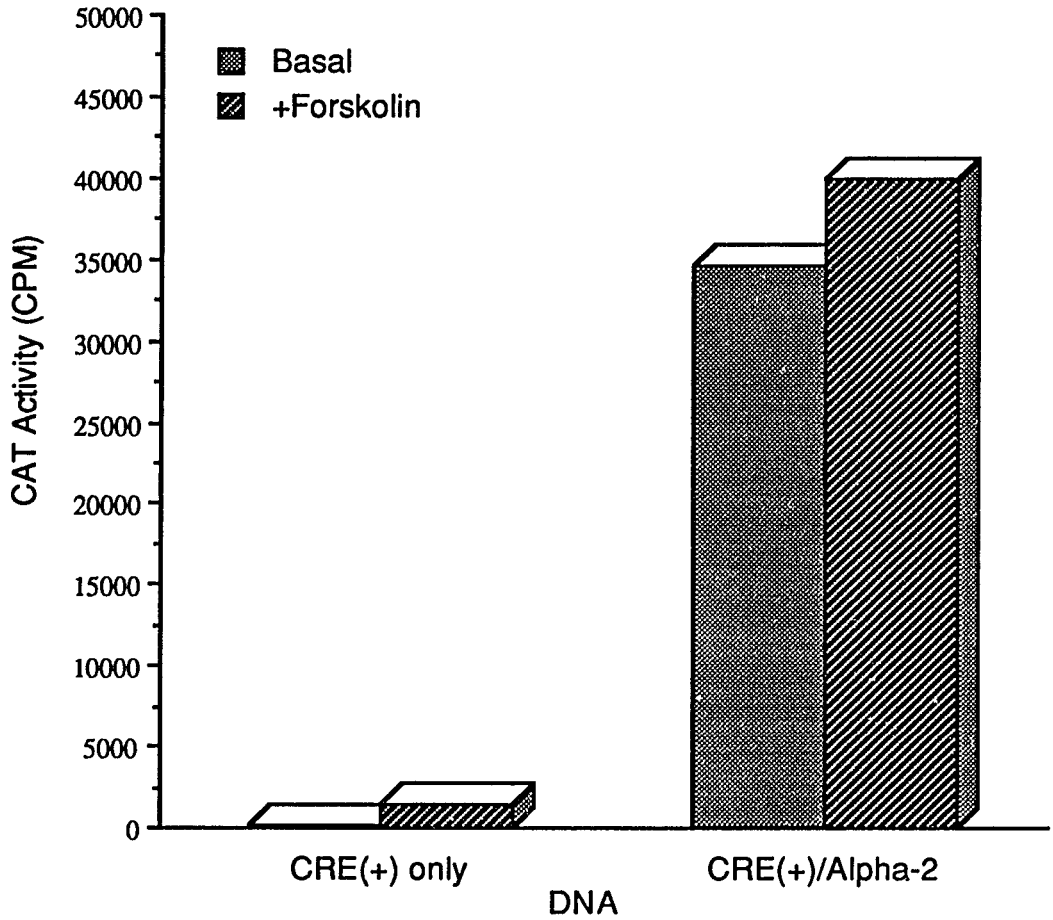


Figure 4. Basal and forskolin-stimulated CAT activity in COS-7 cells transfected using the DEAE-dextran technique. Each bar represents a single plate of cells transfected with either 10 μg of reporter plasmid pCRE(+) alone or 10 μg of reporter plasmid plus 10 μg of α_2 adrenergic receptor coding plasmid.

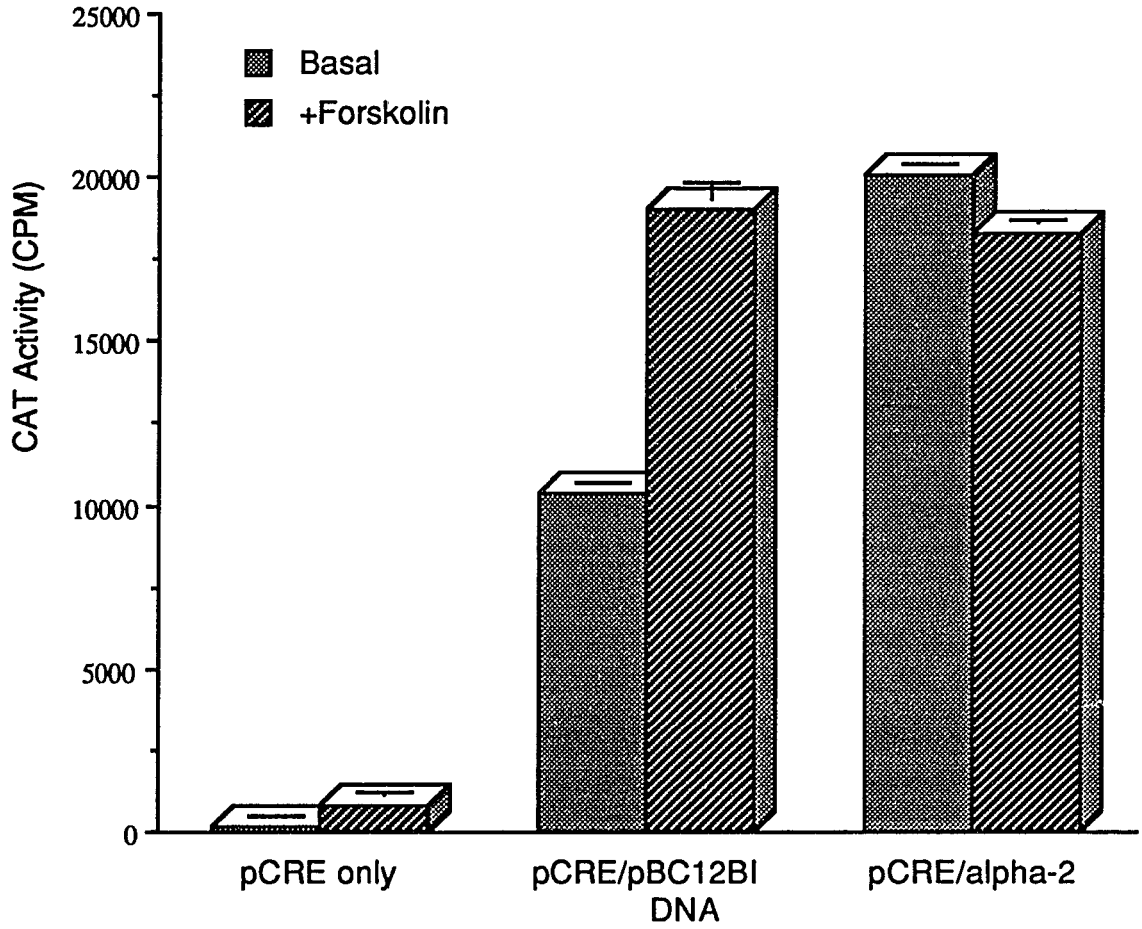


Figure 5. Basal and forskolin-stimulated CAT activity in COS-7 cells transfected with either reporter plasmid pCRE(+) alone, pCRE(+) plus vector pBC12 DNA or pCRE(+) plus pBC12α_2-C10 coding plasmid. Each bar represents the mean $\pm$ S.E. of two individually-transfected plates.

2.3.2 Transient Expression in JEG-3 Cells

Human choriocarcinoma (JEG-3) cells were obtained from American type culture collection and used for subsequent studies with the reporter plasmid. Previous studies of cAMP-dependent promoters have shown that this cell line responds strongly to changes in cAMP (Delegeane *et al.*, 1987). In order to determine if α_2 adrenergic receptors were expressed endogenously in these cells, radioligand binding using [^{3}H]rauwolscine was first performed on membranes from untransfected JEG-3 cells. No specific radioligand binding was observed (data not shown), indicating that JEG-3 cells do not endogenously express α_2 adrenergic receptors. Following calcium phosphate transfection, each of the α_2-adrenergic receptor subtypes could be transiently expressed in JEG-3 cells at levels ranging from 0.2-2 pmol/mg protein. For the three human α_2 receptor subtypes, expression levels in JEG-3 cells were as follows: α_2-C2, 0.75 ± 0.23; α_2-C4, 0.31 ± 0.05; α_2-C10, 1.16 ± 0.33 pmol/mg protein (n=6).

Reporter plasmid was then transfected into these cells in order to measure a functional response. Plates of JEG-3 cells were transfected with the reporter plasmid and incubated with 100 µM forskolin for 16 hours. When compared to the response observed using COS-7 cells (Figure 6), JEG-3 cells exhibited a marked increase in reporter gene expression. Basal activity was over 5 times that observed in COS cells, and forskolin stimulation was over 13-fold, compared to only 4.6 fold in COS. These results imply that the JEG-3 cells respond much more strongly to elevated cAMP than do COS-7 cells.

Subsequent studies involved co-transfection of the α_2-C10 adrenergic receptor and reporter plasmid, followed by a 16-hour incubation with 100 µM

forskolin and α_2 receptor agonists (data not shown). Using this approach, agonist-mediated inhibition of forskolin-stimulated CAT activity was not consistently observed. Therefore, shorter drug incubations using 10 μM forskolin were performed to decrease the stimulatory effect of forskolin (Figure 7). Basal and forskolin-stimulated CAT activity was then assessed in transfected cells following either a 4, 6 or 8-hour incubation. While the basal activities were similar in each case, 10 μM forskolin elicited a time-dependent stimulation of CAT expression. Four-hour incubation with forskolin elicited an 8-fold stimulation, while at six hours stimulation increased only to 9-fold. At 8 hours however, stimulation increased to 14-fold, demonstrating that forskolin-stimulation of CAT activity in transfected cells is dependent on the incubation time. For all subsequent studies, unless indicated, a 4-hour drug incubation time was used to measure α_2-adrenergic receptor function.

Preliminary experiments also demonstrated that the presence of serum (fetal bovine serum, Hyclone Labs.) in the incubation media can attenuate the inhibition obtained with α_2-receptor agonist. Serum, as purchased from the company, contains a number of factors including hormones, vitamins and growth factors. Since serum may also contain sizable amounts of epinephrine, an endogenous α_2 receptor agonist, drug incubations were performed in the absence of serum to eliminate any potential effect of endogenous catecholamines. In the presence of 5% serum, 1 nM medetomidine (a potent α_2 receptor agonist) elicited a 49% inhibition of forskolin (1μM)-stimulated CAT activity, whereas in serum-free media this concentration of medetomidine inhibited 77% of the forskolin-stimulated

activity. These results suggest then, that removal of serum from the drug incubations makes the cells more sensitive to exogenously-added agonist.

In order to demonstrate that these cells respond appropriately to elevated cAMP, a forskolin dose-response curve was performed. Figure 8 shows that forskolin caused a significant dose-dependent stimulation of CAT activity with a maximum 6-fold stimulation occurring at 100 μM forskolin. Other experiments showed that a concentration of 1 μM forskolin, which stimulated CAT activity ~4 fold above basal levels, was optimal in terms of studying the inhibition of CAT activity by co-incubation with α_2-agonists. At higher concentrations of forskolin (10-100 μM), the α_2-mediated inhibition of CAT activity was difficult to observe and thus 1 μM forskolin was used for all subsequent experiments.

These preliminary studies have demonstrated that α_2 adrenergic receptors can be transiently-expressed in human choriocarcinoma cells at reasonably high levels (0.2-2 pmol/mg). For functional studies with the JEG-3 cells, we have used a [^{3}H]chloramphenicol acetyl transferase (CAT) assay (Seed and Sheen, 1988) as a transient measure of intracellular cAMP. Incubation of transfected JEG-3 cells with forskolin produced a dose-dependent increase in CAT activity, implying that the reporter plasmid indeed responds to elevated levels of cAMP. These studies have also demonstrated that using a short, 4-hour incubation with 1 μM forskolin was optimal in terms of observing agonist-mediated inhibition of CAT activity.

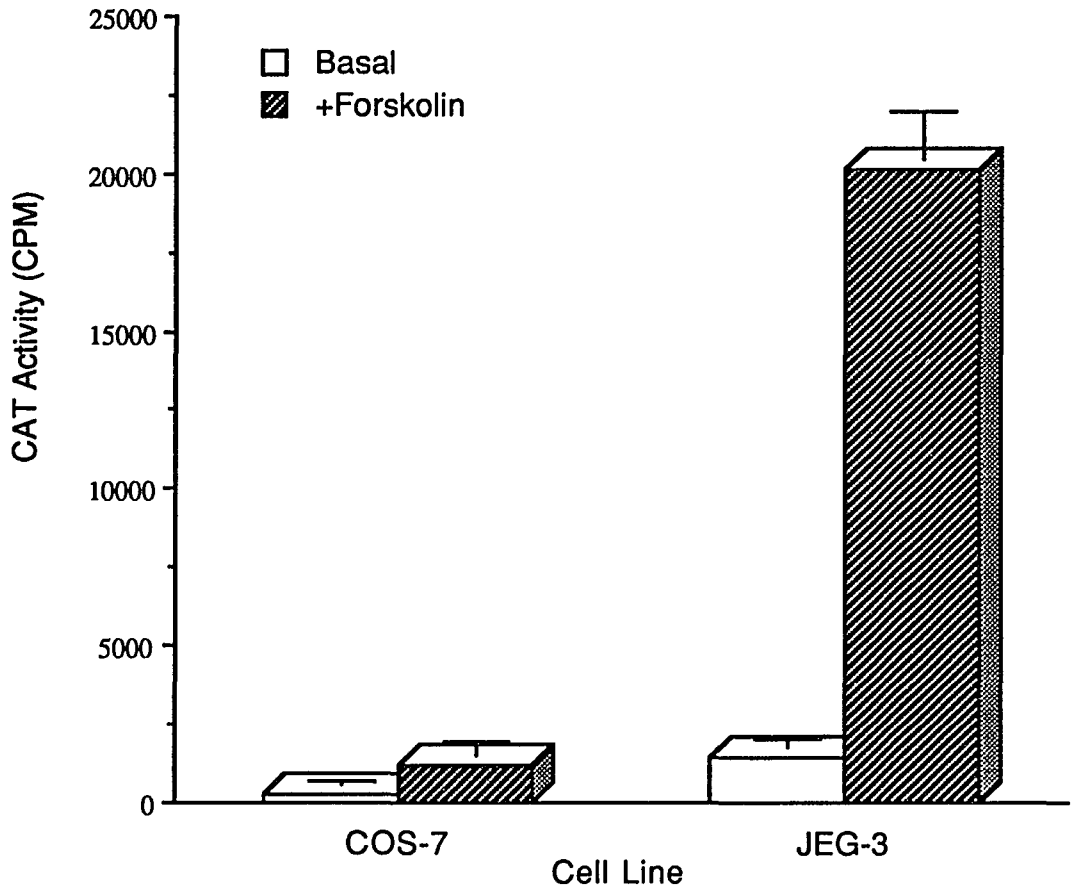


Figure 6. Basal and forskolin-stimulated CAT activity in both COS-7 and JEG-3 cells transfected using calcium phosphate precipitation. Each bar represents the mean ± S.E. of two plates transfected with 10 µg of pCRE(+) reporter plasmid.

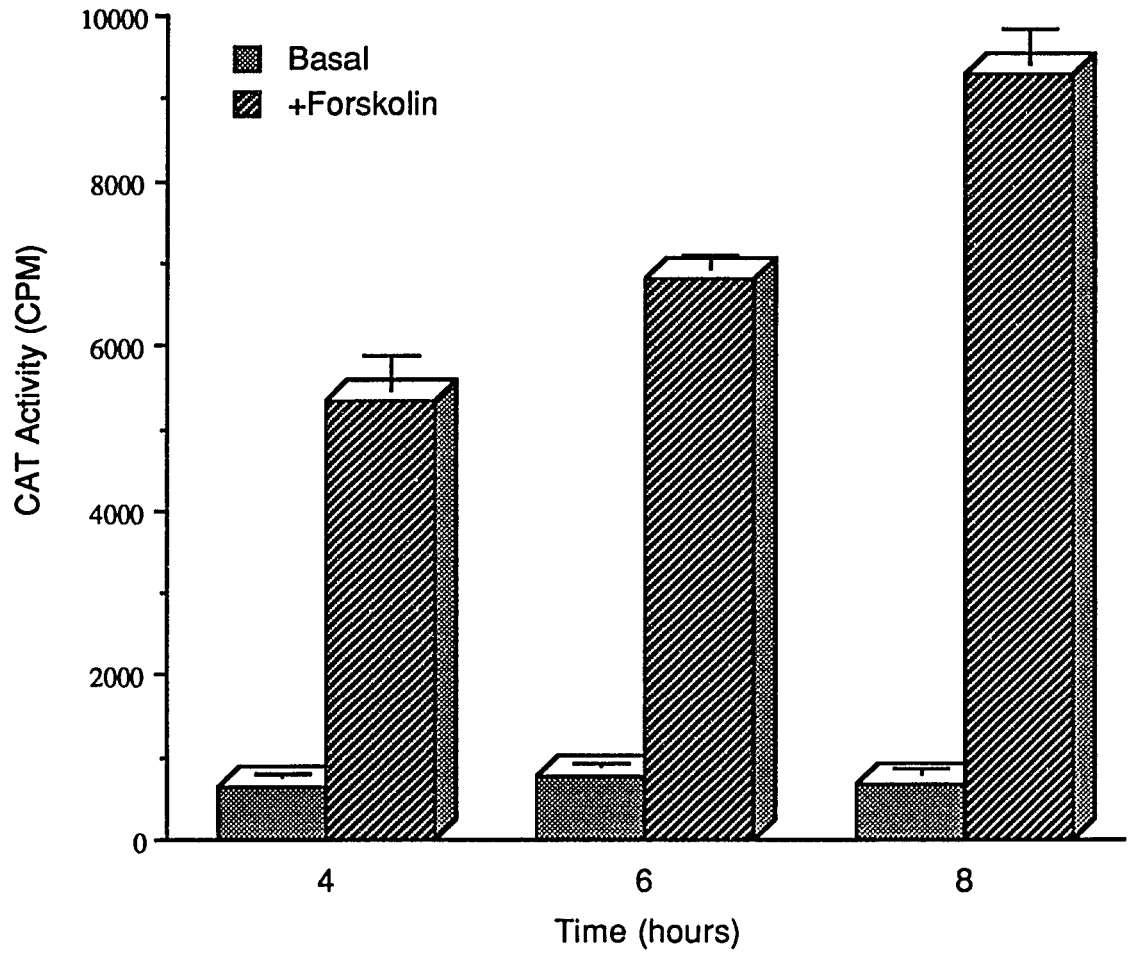


Figure 7. Effect of incubation time on forskolin stimulation of CAT activity in JEG-3 cells transfected with the reporter plasmid pCRE(+). Each bar represents the mean ± S.E. for two individually transfected plates.

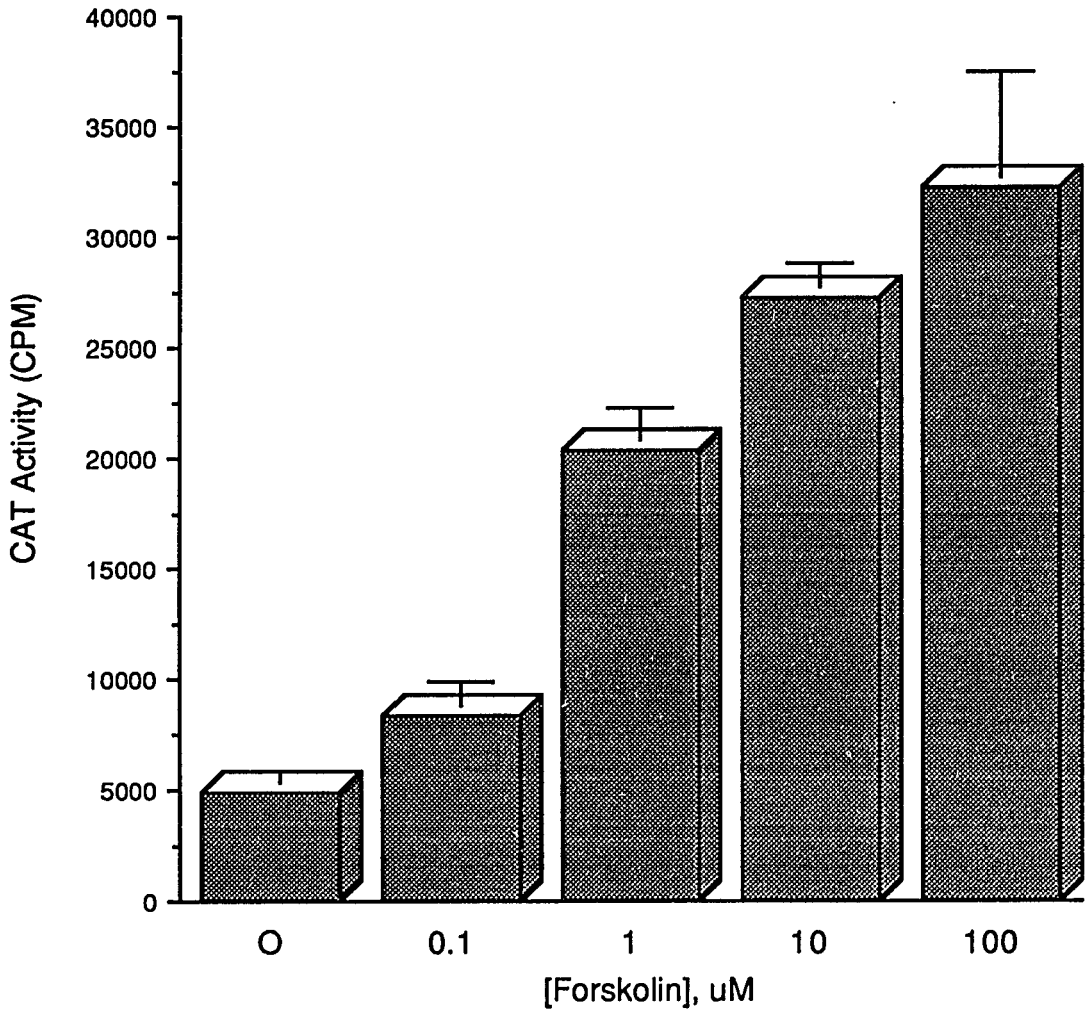


Figure 8. Forskolin stimulation of CAT activity in JEG-3 cells transfected with reporter plasmid and plasmid DNA encoding the α_2-C10 adrenergic receptor. Each bar represents the mean $\pm$ S.E.M. for three individually-transfected plates.

2.4 Discussion

The α_2 adrenergic receptors were among the first members of the G-protein coupled receptor superfamily to be cloned and extensively characterized. (Kobilka *et al.*, 1988). Within the cell, these receptors typically inhibit adenylyl cyclase by interacting with an inhibitory G-protein, G_i (Pepperl and Regan, 1993b). While many of the intracellular effectors to which these receptors couple have also been extensively characterized (G-proteins, adenylyl cyclase, phospholipase C, etc.), Preparation of both site-directed (Wang *et al.*, 1991) and chimeric (Kobilka *et al.*, 1988) mutant α_2 adrenergic receptors has helped define specific regions of the receptor involved in both ligand binding and effector coupling.

With regard to α_2 adrenergic receptor function, the COS-7 cells have proven extremely valuable in terms of studying receptor pharmacology. These cells are very receptive to transfection and are capable of producing large amounts of receptor protein from plasmid DNAs containing an SV40 origin of replication. Typically 10-20% of the cells become transfected and express very high levels of receptor (Cullen, 1987). Since transient expression in COS cells offers a rapid means for producing large amounts of receptor protein, this approach is most commonly used for studying the radioligand binding properties and pharmacology of cloned receptor subtypes (Cotecchia *et al.*, 1990).

Whereas radioligand binding studies principally require only large amounts of receptor protein, functional studies demand that these receptors be coupled to 2nd messenger pathways as well. In order to measure a

functional response to receptor activation, the receptors must be capable of interacting with the intracellular signal transduction machinery. In COS cells, the extremely high level of receptor expression per cell can effectively swamp the available G-proteins and other intracellular effectors. Therefore, a large number of receptors will not be functionally coupled. In terms of studying coupling to stimulation of adenylyl cyclase or phospholipase C activity, this poses no problem. However, since α_2 adrenergic receptors typically couple to inhibition of adenylyl cyclase (Pepperl and Regan, 1993a), one must first stimulate the enzyme with forskolin. The addition of forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1986), to transiently-transfected COS cells will stimulate cAMP production in all cells, whereas only transfected cells are capable of responding to agonist. Thus, transient expression in COS cells is not routinely used for studying the signal tranduction of cloned α_2 adrenergic receptors. Rather, other groups have turned to stable gene expression for studying inhibition of adenylyl cyclase by α_2 adrenergic receptors (Jansson *et al.*, 1994). While stable gene expression systems have been extensively used to study α_2 receptor function, selection and development of such cell lines can take weeks or even months. This lengthy selection process is required to assure that all cells in the population express comparable levels of receptor protein. In this fashion, one can be assured that every cell in the population is capable of responding functionally to agonist. Thus, in order to determine if a newly cloned receptor or mutant receptor still couples to inhibition of adenylyl cyclase, one must typically prepare stably-expressing cell lines. One goal of this work then, was to develop a transient cAMP-responsive gene expression system which would facilitate the study of

cloned α_2 adrenergic receptor function. In this manner, we might quickly determine the coupling of a new receptor clone or the effect of a mutation on receptor function. Such a transient system could also prove potentially useful for any receptor coupled to the adenylyl cyclase signal transduction pathway.

We initially chose the COS cells for development of a transient, functional system. First, these cells are an immortalized simian kidney cell line which will grow continuously in culture. Secondly, COS cells are easy to transfect, and following transfection, will express very high levels of adrenergic receptor protein (Regan *et al.*, 1988). Finally, the pharmacology of the α_2 adrenergic receptor subtypes has been extensively examined following transient expression in the COS cells (Bylund *et al.*, 1992). We therefore postulated that these cells would provide a suitable host for development of a functional transient expression system.

The introduction of reporter plasmid TESBglIICRE(+)ΔNHSE into COS-7 cells using calcium phosphate-mediated transfection elicited little basal CAT activity and only a small forskolin stimulation, even following a 16-hour incubation. One important observation was that co-transfection of cells with DNA encoding the α_2-C10 adrenergic receptor subtype elicited a marked increase in basal CAT activity with no observable forskolin stimulation. This effect was greatly enhanced following DEAE-dextran transfection of the COS cells. Since transfection efficiency may be much higher (about 10-fold) using the DEAE-Dextran method (Cullen, 1987), a larger portion of cells became transfected and exhibited this enhanced activity. This enhanced activity was still observed following transfection of cells with reporter plasmid and vector pBC12 DNA lacking the receptor coding sequence. Moreover, this unusually

high activity was also evident following transfection of the COS cells with reporter plasmid lacking a CRE (TESΔNHSE). From these results we can conclude that this unusual activity is not dependent on the CRE, CREB protein or the α_2 receptor inself. The JEG-3 cells, which cannot replicate plasmid DNAs like COS cells, did not exhibit such enhanced activity following co-transfection of reporter plasmid and pBC12BI. Therefore it would appear that this effect is unique to the COS cells themselves. This unusual stimulatory effect may in fact, be related to the COS cells' unique ability to replicate plasmid DNAs to a high copy number. One possible explanation is that the large amount of plasmid DNA produced in the transfected COS cell population is acting to enhance expression from the tk-CAT promoter. A second possibility is that some product of the pBC12 vector DNA, such as the amp gene product (β-lactamase) stimulates expression from the tk-CAT promoter. A third explanation is that presence of the SV40 ori in the pBC12 plasmid also stimulates high-level transcription from the CAT promoter. In any case, one potential solution would involve elimination of the SV40 ori in the pBC12BI plasmid. This would prevent high-level plasmid replication, and might offer one approach to understanding the molecular biology of this unusual phenomenon.

Since the COS cells did not elicit suitable levels of reporter gene expression, we turned to the JEG-3 cells for development of a functional assay system. These cells were initially derived from a human placental tumor, and express both the α and β subunits of chorionic gonadotropin. (Kohler and Bridson, 1971). In addition, the JEG-3 cells were shown to possess a tissue-specific enhancer which boosts expression of genes regulated by CREs.

(Delegeane *et al.*, 1987). Since then, these cells have provided a model system for study of cAMP-responsive genes (Collins *et al.*, 1990).

The CAT reporter plasmid used in our studies TESBglIICRE(+)ΔNHSE is virtually identical to that used by Delegeane *et al.* (1987). This plasmid contains the CRE from the gene for the α-subunit of human chorionic gonadotropin. Following calcium phosphate transfection, it became immediately apparent that cAMP-dependent CAT expression was substantially higher in JEG-3 cells relative to COS-7 cells (figure 6). This in part was due to the length of forskolin incubation. In similar experiments involving dopamine receptors, Montminy and Borrelli (1991) used relatively long (10-12 hour) incubations with drug to achieve stimulation of CAT activity. In addition, our preliminary results with COS-7 cells indicated that similar long incubations with relatively high doses (100 μM) of forskolin were necessary to observe stimulation of CAT activity. However it became clear that shorter incubations (4 hours) with much lower doses of forskolin (1 μM) would be adequate to observe stimulation of CAT activity in transfected JEG-3 cells. Higher doses of forskolin also made it difficult to consistently observe inhibition of CAT activity by co-expression and activation of α_2 adrenergic receptors. Similarly, incubating the transfected cells with 1 μM forskolin for less than four hours did not elicit consistently detectable levels of reporter gene expression (data not shown).

The CAT assay system used for these studies also differs from that of Montminy and Borrelli (1991), in that their system utilized a [^{14}C]CAT assay (Gorman *et al.*, 1982) to measure reporter gene activity. While this has been the traditional approach for measuring CAT expression, we found that a

[^{3}H]CAT assay system was much more amenable to the large-scale experiments performed here. In contrast to the [^{14}C]CAT system, the [^{3}H]CAT assay developed by Seed and Sheen (1988) is both faster and does not require chromatography to separate away the product chloramphenicol.

While addition of forskolin (1μM) was adequate to stimulate expression of CAT activity, other approaches are still potentially available for stimulating reporter gene expression. One approach involves co-transfecting the JEG-3 cells with not only reporter plasmid and α_2 adrenergic receptor coding plasmid, but also plasmid encoding a receptor which stimulates adenylyl cyclase, such as β-adrenergic receptor. Although this would more closely mimic the native system, this approach is logistically more complex due to the addition of a third transfecting plasmid. Moreover, addition of forskolin (1 μM) elicited a consistent 3-5 fold increase in CAT gene expression, regardless of basal activity. Although forskolin will stimulate cAMP production in the entire cell population, only those cells taking up the reporter plasmid will be capable of responding to agonist by expressing CAT activity. Cells lacking reporter plasmid then, are effectively invisible. The present system works since the same population of cells takes up both the reporter plasmid and the α_2 receptor coding plasmid. Any cell possessing reporter plasmid should also be expressing α_2 adrenergic receptor and hence, capable of responding to agonist.

CHAPTER 3

PHARMACOLOGY OF α_2 ADRENERGIC RECEPTORS EXPRESSED IN JEG-3 CELLS

3.1 Introduction

The pharmacology of the cloned α_2 receptor subtypes has been extensively studied following transient expression in COS-7 cells (Bylund *et al.* 1992). Initially defined as the pharmacologic α_{2A} receptor subtype (Bylund, 1985), the α_2C10 receptor possesses high affinity for the antagonists rauwolscine, yohimbine and phentolamine (table 1). Like the native platelet α_2 receptor, the α_2-C10 subtype exhibits a rather low affinity for the α_1 antagonist prazosin, and surprisingly low affinities for the endogenous catecholamines epinephrine and norepinephrine. The agonist oxymetazoline however, binds much more selectively to the α_2-C10 subtype compared to the other human α_2 receptor subtypes (Lomasney *et al.*, 1990).

The α_2-C2 receptor subtype appears to correspond to the pharmacologically-defined α_{2B} adrenergic receptor (Bylund *et al.*, 1992). This subtype binds the antagonists rauwolscine and phentolamine with high affinity similar to the α_2-C10. This receptor also displays intermediate affinity for prazosin (K_i = 293 nM), but a much lower affinity for oxymetazoline (K_i = 1500 nM) compared to the α_2-C10 subtype. Oxymetazoline is perhaps one of the only compounds which can differentiate between all three subtypes, having the highest affinity for the α_2-C10, followed by the α_2-C4 with the lowest affinity for the α_2-C2 subtype. Most other agonists tested bound the α_2-C2

with low affinities similar to oxymetazoline, except for p-aminoclonidine, which was over 10-fold more potent (K_i = 120 nM).

Although initially defined as an α_{2B} receptor, the cloned α_2-C4 adrenergic receptor possesses five-fold higher affinity for [^{3}H]rauwolscine and substantially higher affinity for prazosin than the α_2-C10 receptor (Regan *et al.*, 1988). Likewise, the catecholamine agonists epinephrine and norepinephrine were nearly 10-fold more potent at the α_2-C4 subtype. The overall order of potency for inhibition of [^{3}H]rauwolscine binding to the α_2-C4 was yohimbine > WB4101 > idazoxan > phentolamine > prazosin > oxymetazoline > p-aminoclonidine > epinephrine > norepinephrine. The high affinity for prazosin demonstrated by the α_2-C4 subtype initially prompted its classification as an α_{2B} subtype. Comparison of the radioligand binding properties of the α_2-C4 receptor subtype with the neonatal rat lung α_{2B} subtype and the opossum kidney α_{2C} subtype revealed a much better correlation to the α_{2C} subtype. Both the α_2-C4 and the OK cell α_{2C} receptor possess higher affinity for [^{3}H]rauwolscine than either the α_{2A} or α_{2B} subtypes. Moreover, the agonist oxymetazoline possesses identical affinity for the α_2-C4 and the OK cell α_2 receptor. This affinity appears intermediate to those for the α_{2A} (high) and α_{2B} subtypes (low), suggesting that the α_2-C4 encodes the pharmacologic α_{2C} receptor subtype (Bylund *et al.*, 1992).

The expressed fish α_2 receptor exhibits a pharmacology characteristic of an α_2 receptor subtype. The α_2-F binds [^{3}H]rauwolscine with high affinity (K_D = 0.8 nM), and possesses high affinity for both oxymetazoline and yohimbine. Prazosin binds this receptor with fairly low affinity (K_I = 469 nM), similar to the α_2-C2 receptor. While UK-14304 is an agonist at most α_2

receptors, this compound possesses no intrinsic activity at fish melanophore α_2-receptors (Karlsson *et al.*, 1989).

While these radioligand binding studies have helped clarify the molecular nature of α_2 adrenergic receptor heterogeneity (Bylund et al., 1992), few studies have directly compared the agonist pharmacology of these receptors in a single system. Only recently, Jansson *et al.* directly compared the coupling of the human α_2 adrenergic receptors to cAMP production in stably-transfected mouse S115 mammary tumor cells (Jansson *et al.*, 1994). In a similar manner, it was our goal to use transient expression in JEG-3 cells to compare both agonist potency and efficacy in a single system. Using the conditions defined in chapter two, we proceeded to examine the effects of various adrenergic agonists on forskolin-stimulated CAT expression in transiently-transfected JEG-3 cells.

TABLE 1

K_I VALUES FOR COMPETITION BY ADRENERGIC LIGANDS FOR BINDING OF [^{3}H]YOHIMBINE[1] OR [^{3}H]RAUWOLSCINE[2] TO MEMBRANES PREPARED FROM COS-7 CELLS EXPRESSING α_2 ADRENERGIC RECEPTOR SUBTYPES

	Ki (nM)			
COMPOUND	$^1\alpha_2$-C2	$^1\alpha_2$-C4	$^1\alpha_2$-C10	$^2\alpha_2$-F
rauwolscine	11	2.1	7.1	0.3
phentolamine	9.2	14.4	6.2	104
oxymetazoline	1506	125	13.2	49
epinephrine	851	318	1671	ND
norepinephrine	1265	606	3677	2849
p-aminoclonidine	120	97	31	92
prazosin	293	67.7	2237	469

[1] From Lomasney *et al.*, 1990.
[2]FromSvensson *et al.*, 1993.
ND=Not determined.

3.2 Results

3.2.1 α_2-C10

Since our initial studies involved the α_2-C10 adrenergic receptor, we proceeded to first characterize functional responses to activation of this receptor subtype. Radioligand binding studies using [^{3}H]rauwolscine demonstrated that using the calcium phosphate precipitation technique, the α_2-C10 receptor subtype could be transiently expressed at 0.5-1.5 pmol/mg protein. In order to determine the efficiency of transfection, JEG-3 cells were propagated on glass cover slides, transfected with α_2-C10 plasmid DNA, and subsequently probed with antibodies directed against the third intracellular loop of the receptor (Vanscheeuwijck *et al.*, 1993). Probing of α_2-C10 transfected cells with α_2-C10 specific antibodies demonstrated that approximately one in 100 cells becomes transfected and expresses α_2 receptor (data not shown). For the α_2-C10 receptor this labelling appeared mainly on the plasma membrane and concentrated in isolated patches within the nucleus. Since the overall transfection efficiency is rather low, this suggests that the actual level of expression within the transfected cell population is substantially higher.

We then examined the effect of various adrenergic agonists on forskolin-stimulated CAT activity in α_2-C10 transfected JEG-3 cells. The first agonists examined at this receptor were epinephrine and medetomidine (figure 9). Epinephrine is the endogenous non-selective catecholamine agonist, whereas medetomidine is a potent α_2-selective imidazoline agonist. (Virtanen *et al.*, 1988). Figure 10 shows dose-response curves for the effects

of α_2-agonists medetomidine and epinephrine on forskolin-stimulated CAT activity in JEG-3 cells co-transfected with plasmids encoding the α_2-C10 receptor subtype and the reporter gene (Pepperl and Regan, 1993b). These compounds represent the two primary classes of α_2 adrenergic receptor agonists. Both agonists elicited a bi-phasic response in α_2-C10 transfected cells, with low concentrations of agonist inhibiting CAT activity and high concentrations either returning activity to the 100% level (medetomidine) or increasing activity ~1.4 fold to 240% (epinephrine). In each case the maximal inhibition obtained was ~60% and high doses of medetomidine would merely reverse the inhibition observed at lower doses, whereas micromolar doses of epinephrine would consistently produce a distinct potentiation of the forskolin-stimulated activity.

Other agonists were also tested at the α_2-C10 receptor expressed in JEG-3 cells. Like epinephrine and medetomidine, each agonist elicited a bi-phasic response in JEG-3 cells transfected with the α_2-C10 adrenergic receptor. Figure 12 shows the dose response curves for agonists p-aminoclonidine and UK-14304. These compounds, both α_2-selective agonists, produced a similar dose-dependent inhibition of forskolin-stimulated CAT activity followed by a dose-dependent reversal at higher doses of drug. p-Aminoclonidine was among the most potent drugs at inhibiting forskolin-stimulated CAT activity, with an EC_{50} value of only 0.06 nM, whereas UK-14304 was nearly 100-fold less potent (EC_{50} = 6.3 nM). Again, each drug produced ~60% inhibition of forskolin-stimulated CAT activity followed by a reversal similar to medetomidine. It is particularly interesting that while the catecholamines epinephrine and norepinephrine would produce a clear

potentiation of forskolin-stimulated CAT activity at the higher doses, imidazoline agonists merely reversed the inhibition, even at the very highest doses.

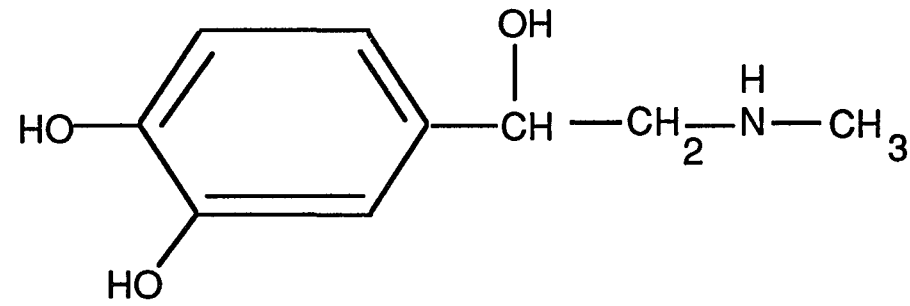
In order to demonstrate that these responses were due to activation of the transiently-expressed α_2 adrenergic receptor, we wished to block these responses pharmacologically using selective adrenergic antagonists. The inhibition induced by 10 nM p-aminoclonidine could be blocked by co-incubation with the α_2-selective antagonist yohimbine, but not by the α_1-antagonist prazosin or the β-selective antagonist propranolol (Figure 13). Similarly, the potentiation of forskolin-stimulated CAT expression induced by 10 μM epinephrine could be selectively blocked by α_2, but not by α_1 or β-adrenergic receptor antagonists (Figure 14). Thus, we have demonstrated that agonist activation of transiently-expressed α_2 adrenergic receptors can modulate expression of a reporter gene linked to cAMP. Furthermore, these functional responses to α_2 adrenergic activation exhibit the pharmacology characteristic of an α_2 receptor subtype.

Since α_2 receptors typically couple to inhibition of adenylyl cyclase via a pertussis toxin sensitive G-protein, we examined the effect of pertussis toxin pretreatment on JEG-3 cells transiently expressing the α_2-C10 receptor subtype. Cells were co-transfected with reporter plasmid and α_2-receptor coding plasmid and then exposed to 100 ng/ml pertussis toxin for 18 hours prior to drug incubation. An epinephrine dose-response curve was performed and the data shown in figure 15. Following pertussis toxin treatment, epinephrine-induced inhibition of forskolin-stimulated CAT activity was abolished. Instead, epinephrine elicited a dose-dependent potentiation of

CAT activity, with a 282% potentiation of forskolin-stimulated CAT activity at the highest dose. In contrast, untreated α_2-C10 transfected cells would elicit only a 100-150% increase at the equivalent dose of epinephrine. This result demonstrates that in transfected JEG-3 cells, the α_2-C10 receptor subtype couples to two independent pathways. One of these is a pertussis toxin-sensitive pathway coupled to inhibition of cAMP production, while the other increases apparent cAMP levels via a pertussis toxin-insensitive mechanism.

To summarize results with the α_2-C10 adrenergic receptor subtype, a number of adrenergic agonists were examined for their ability to modulate forskolin-stimulated CAT activity in transiently-transfected JEG-3 cells. Each agonist produced a bi-phasic response in α_2-C10 transfected cells, with low doses producing inhibition of forskolin-stimulated CAT activity and higher doses either returninig CAT activity to control levels (medetomidine, p-aminoclonidine, UK-14304.) or significantly potentiating CAT activity (epinephrine, norepinephrine). Each of these functional responses could be specifically blocked by α_2, but not α_1 or β adrenergic receptor antagonists. Furthermore, agonist-induced inhibition of forskolin-stimulated CAT activity could be completely attenuated by pre-treatment of transfected cells with pertussis toxin. The potentiation of forskolin-stimulated CAT activity however, was unaffected by pertussis toxin, suggesting that these two responses involve distinct G-protein mediators.

Epinephrine



Medetomidine

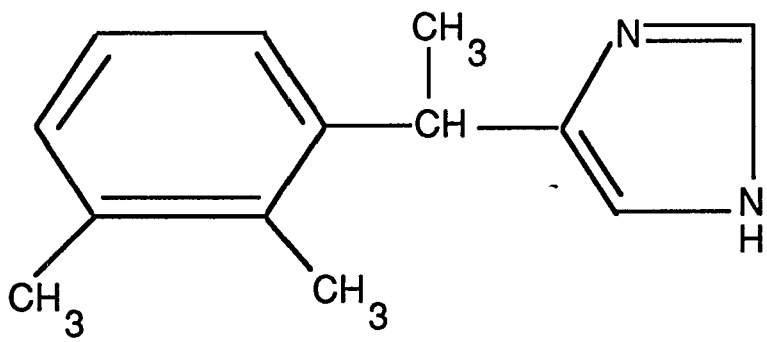


Figure 9. Structures of α_2-adrenergic receptor agonists epinephrine (top) and medetomidine (bottom).

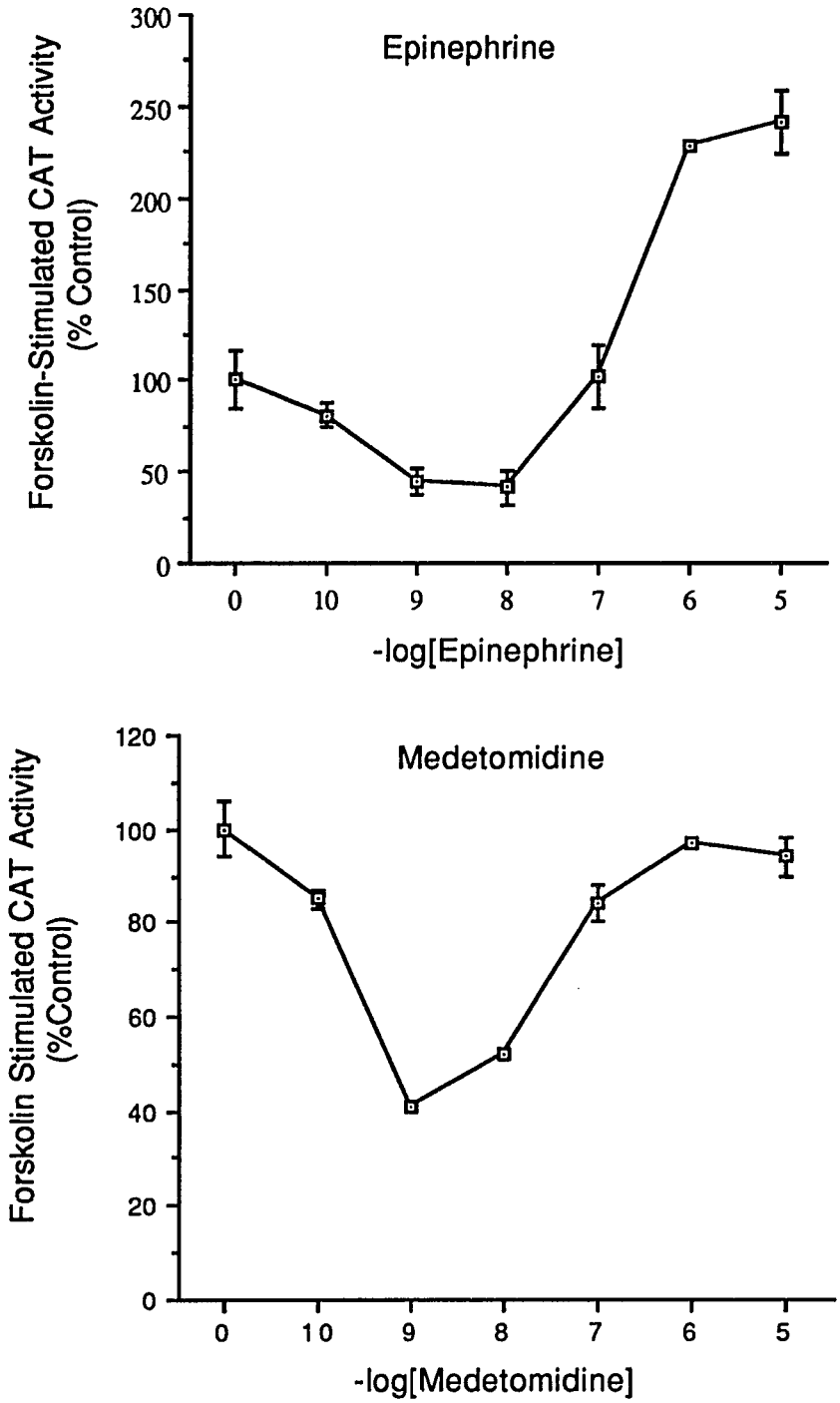


Figure 10. Effect of α_2-adrenergic receptor agonists epinephrine and medetomidine on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C10 DNA.

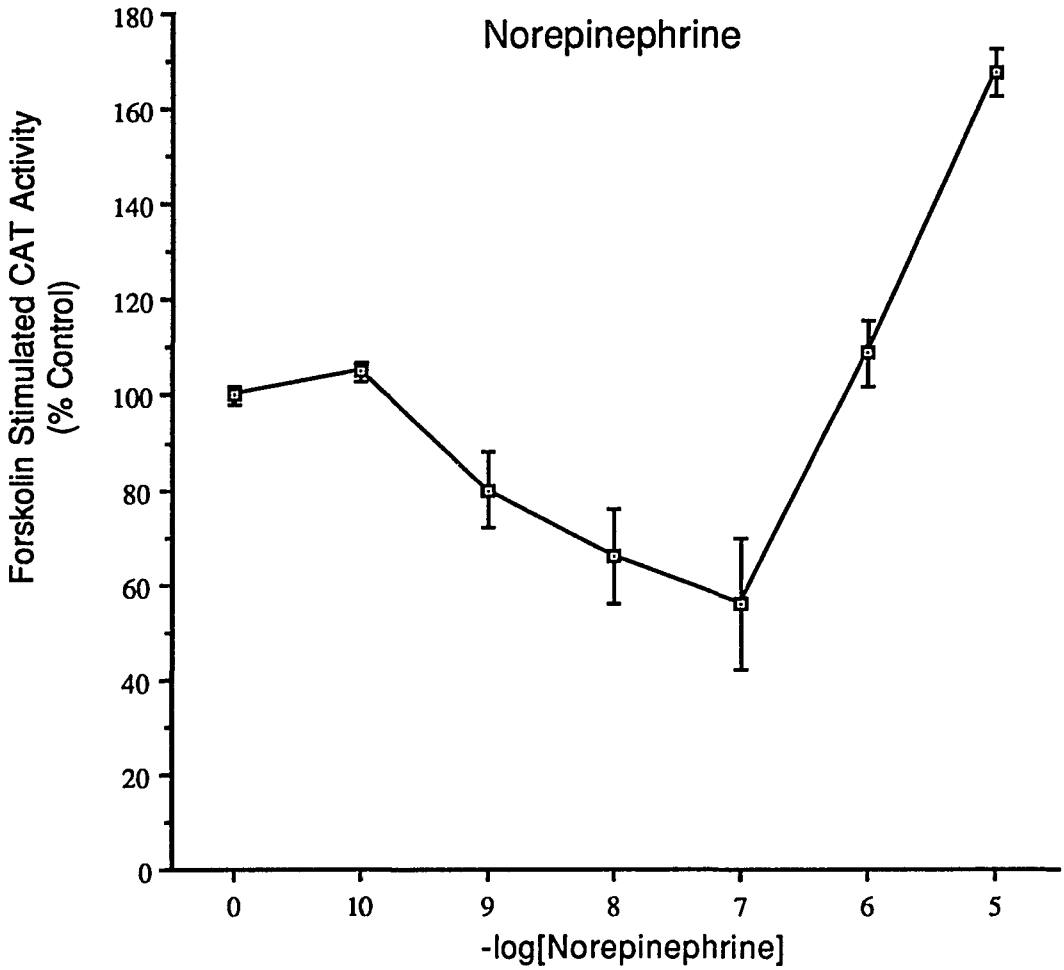


Figure 11. Effect of norepinephrine on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C10 DNA.

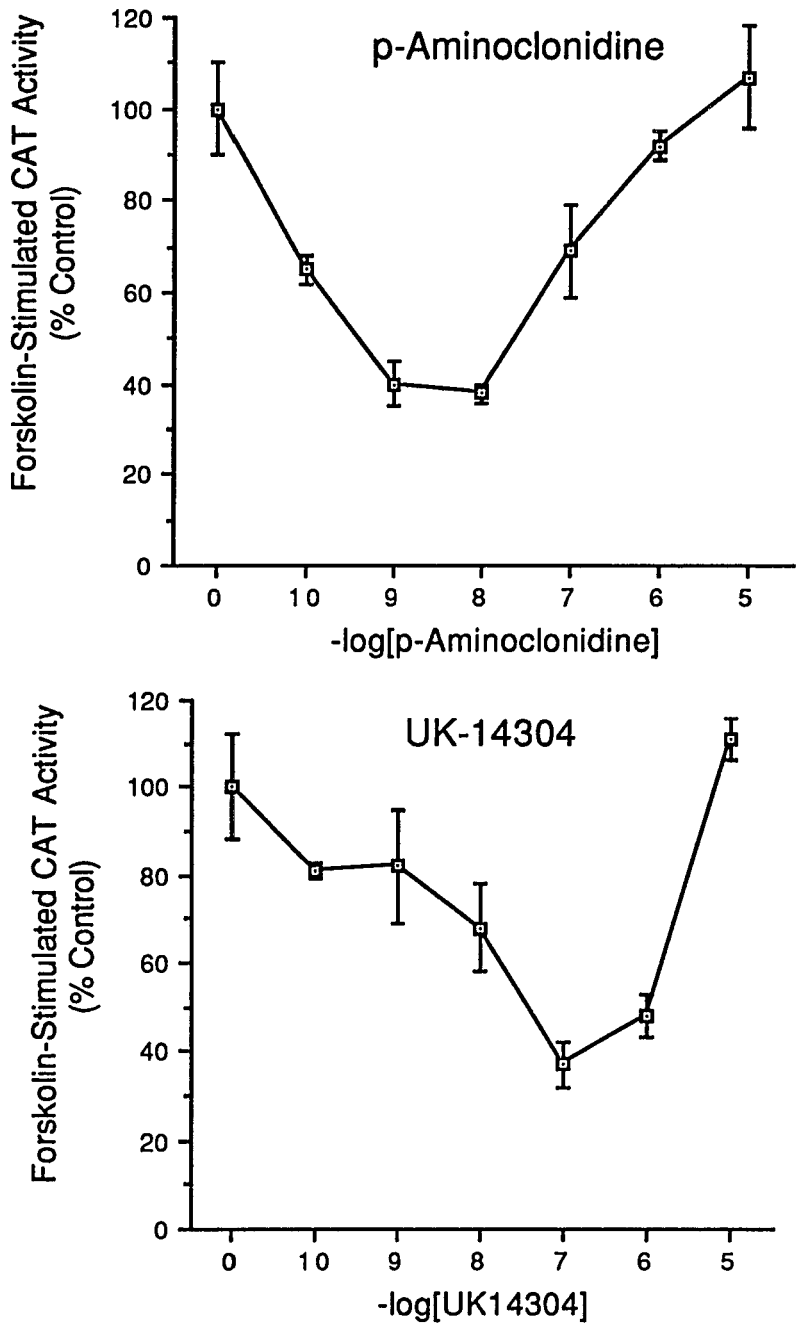


Figure 12. Effect of agonists p-aminoclonidine and UK-14304 on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C10 DNA.

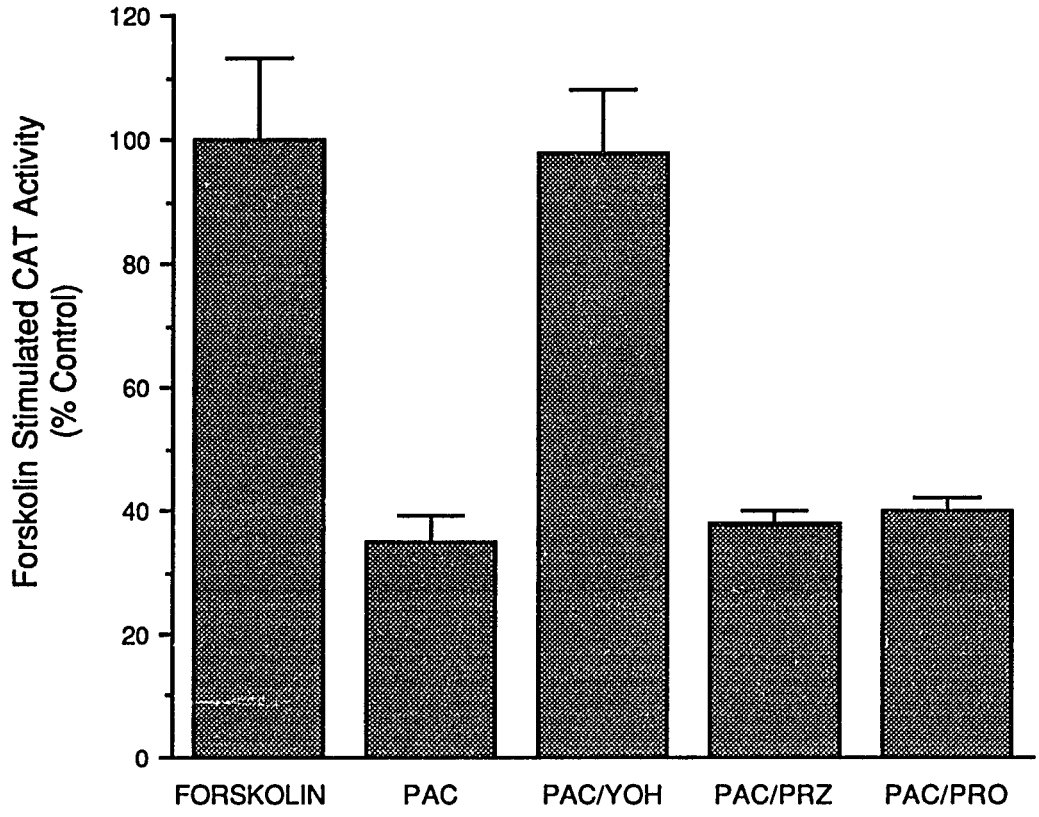


Figure 13. Effect of adrenergic antagonists yohimbine (YOH), prazosin (PRZ) and propranolol (PRO) on the inhibition of CAT activity induced by α_2 adrenergic agonist p-aminoclonidine (100 nM) in α_2-C10 transfected JEG-3 cells. Forskolin and antagonists were added at a 1 μM final concentration.

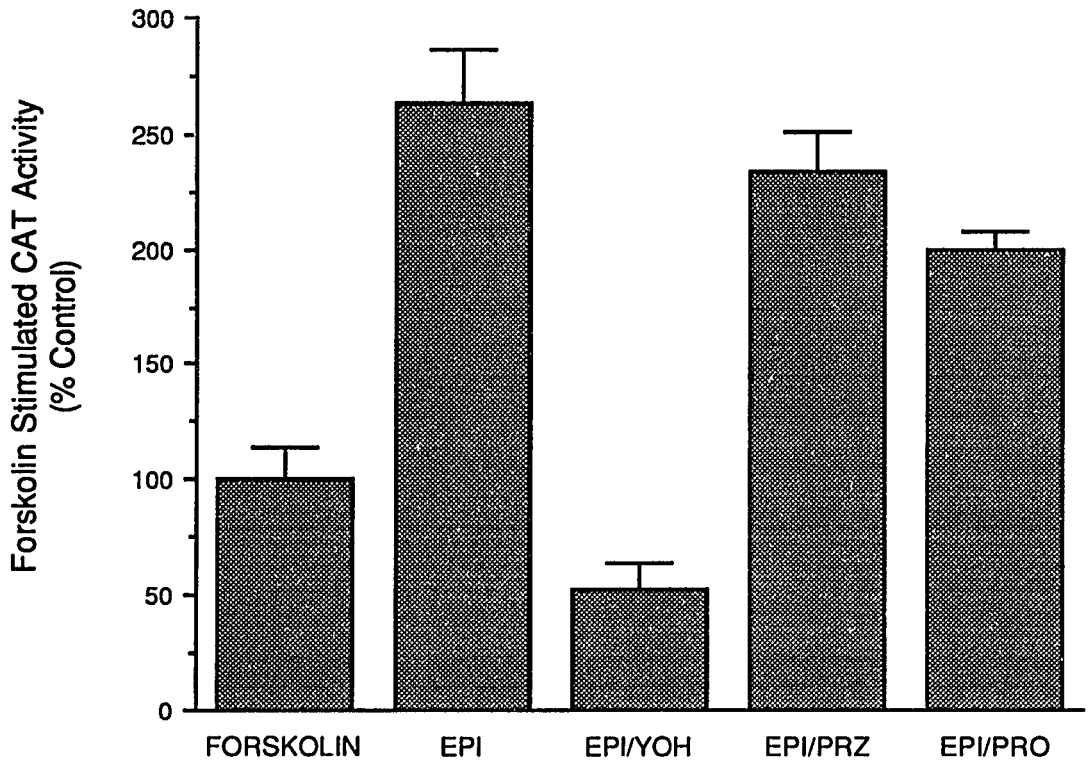


Figure 14. Effect of adrenergic antagonists yohimbine (YOH), prazosin (PRZ) and propranolol (PRO) on the potentiation of CAT activity induced by 10 μM epinephrine in α_2-C10 transfected JEG-3 cells. Forskolin was present at 1 μM final concentration and antagonists were added at 10 μM final concentration.

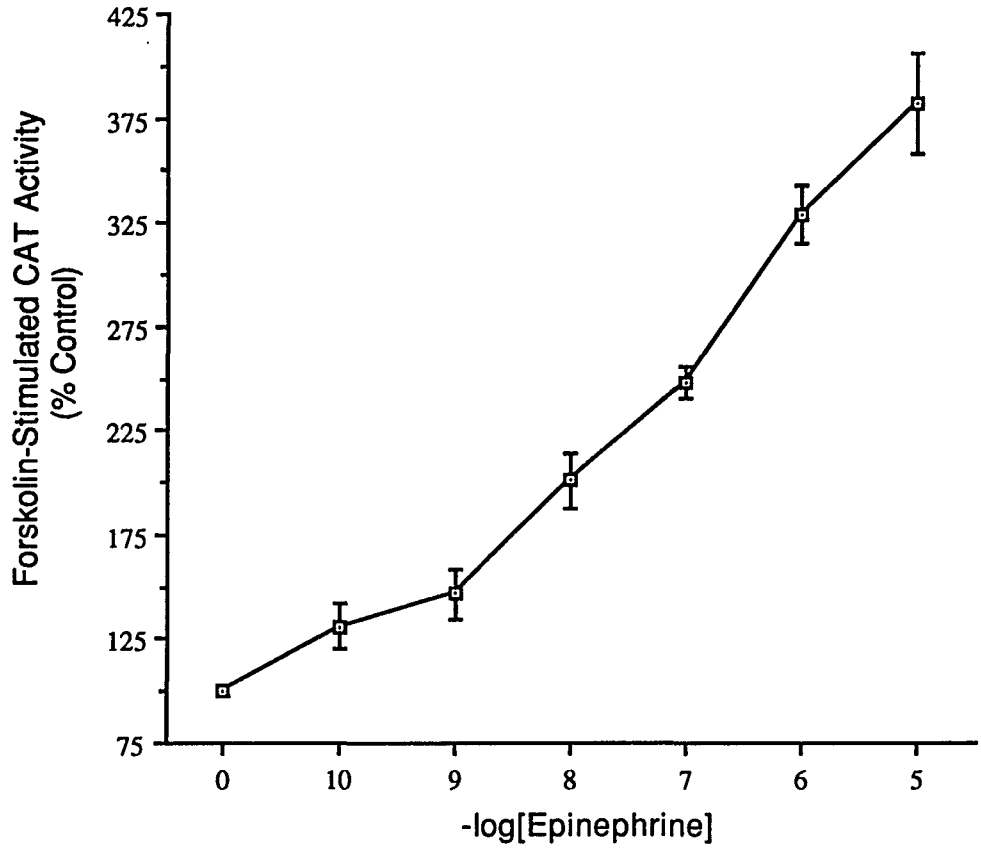


Figure 15. Effect of epinephrine on forskolin-stimulated CAT activity in JEG-3 cells transfected with the reporter plasmid and the α_2-C10 adrenergic receptor coding plasmid and pre-treated for 18 hours with 100 ng/ml pertussis toxin.

3.2.2 α_2-C4

We then assessed functional responses in transiently-transfected JEG-3 cells expressing the α_2-C4 adrenergic receptor subtype. Radioligand binding studies demonstrated that this receptor subtype could be transiently-expressed at 0.3-0.4 pmol/mg in the JEG-3 cells. (n=6). Probing these transfected cells with antibodies selective for the α_2-C4 receptor subtype revealed labelling mainly on the plasma and nuclear membranes. Although the level of expression was lower than for the α_2-C10, the transfection efficiency again was about 0.5-1%.

With respect to functional studies, cells expressing the α_2-C4 receptor subtype displayed a purely inhibitory response to agonist. Figure 16 shows the effects of the agonists epinephrine and medetomidine on forskolin-stimulated CAT activity in transiently-transfected JEG-3 cells expressing the α_2-C4 receptor subtype. Both epinephrine and medetomidine inhibited forskolin-stimulated CAT activity in a dose-dependent manner, with no apparent reversal of inhibition at the higher doses of agonist. Medetomidine was 100-fold more potent in eliciting this response with an EC_{50} of 0.2 nM compared to 20 nM for epinephrine. The agonists p-aminoclonidine and UK-14304 similarly inhibited forskolin-stimulated CAT expression in a dose-dependent manner, with no apparent potentiation of activity at the higher doses of agonist. (figure 17). These compounds were each approxmately 10-fold less potent than medetomidine and 10-fold more potent than epinephrine at this receptor subtype (table 2). Norepinephrine was approximately 10-fold more potent than epinephrine at the α_2-C4 subtype whereas oxymetazoline,

another α_2-selective agonist, was twice as potent as epinephrine (EC_{50} = 10 nM).

In contrast to α_2-C10 transfected cells, each agonist produced nearly complete inhibition of the forskolin-stimulated CAT activity in α_2-C4 transfected cells. This inhibition could be completely attenuated by co-incubation with the α_2 receptor antagonist yohimbine, but not by incubation with either the α_1 receptor antagonist prazosin, or the β adrenergic receptor antagonist propranolol (figure 19). Although prazosin possesses much higher binding affinity for the α_2-C4 receptor subtype compared to the α_2-C10 (Regan *et al.*, 1988), this compound had little effect on functional responses in the JEG-3 cell system. Pretreatment of transfected JEG-3 cells with pertussis toxin completely ablated the inhibitory effect of epinephrine on forskolin-stimulated CAT activity and instead, a slight, but significant potentiation of CAT activity was observed (figure 20). This result suggests that in contrast to the α_2-C10 adrenergic receptor, the α_2-C4 receptor subtype does not couple significantly to the cAMP-stimulatory pathway in JEG-3 cells. Rather, this receptor appears to couple exclusively to the inhibition of adenylyl cyclase via a pertussis-toxin sensitive G-protein.

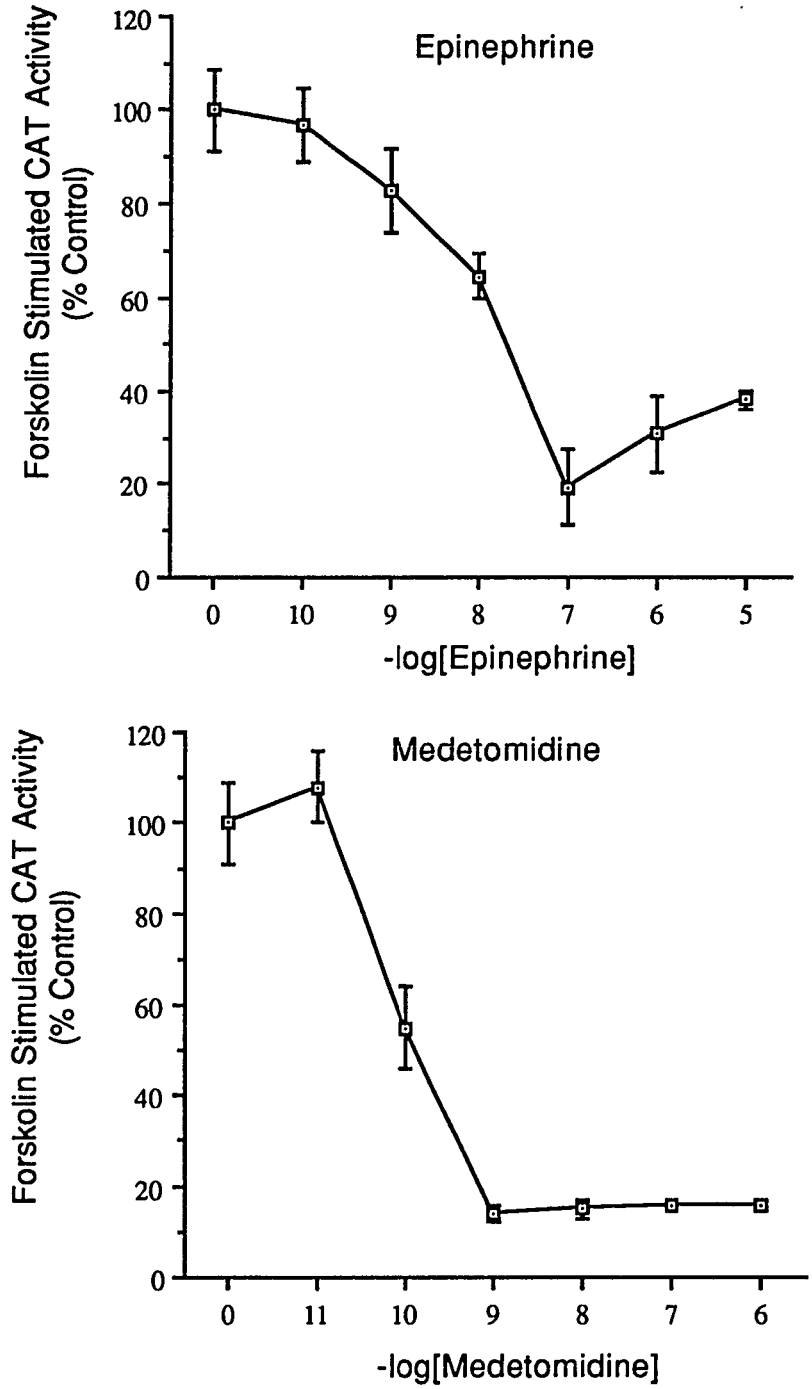


Figure 16. Effect of α_2-adrenergic receptor agonists epinephrine and medetomidine on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C4 DNA.

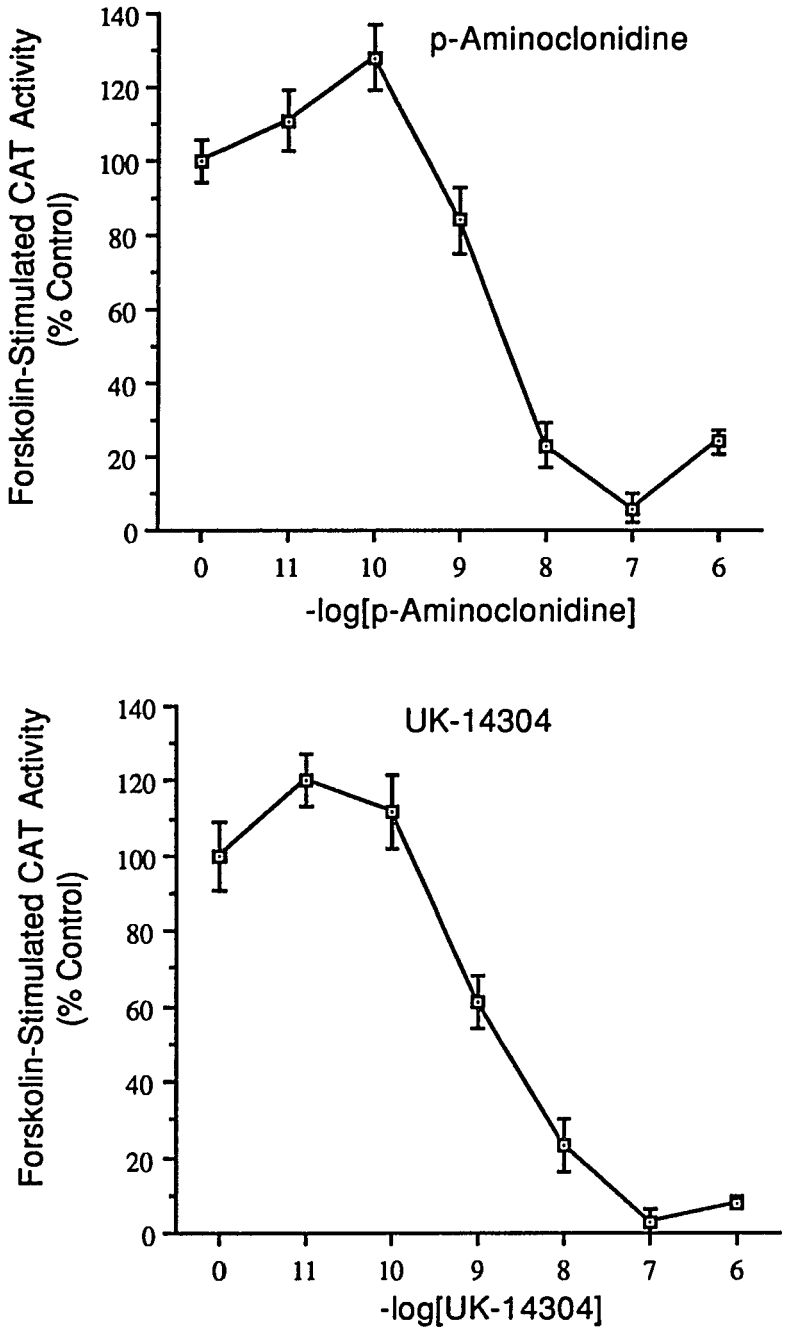


Figure 17. Effect of α_2-adrenergic receptor agonists p-aminoclonidine and UK-14304 on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C4 DNA.

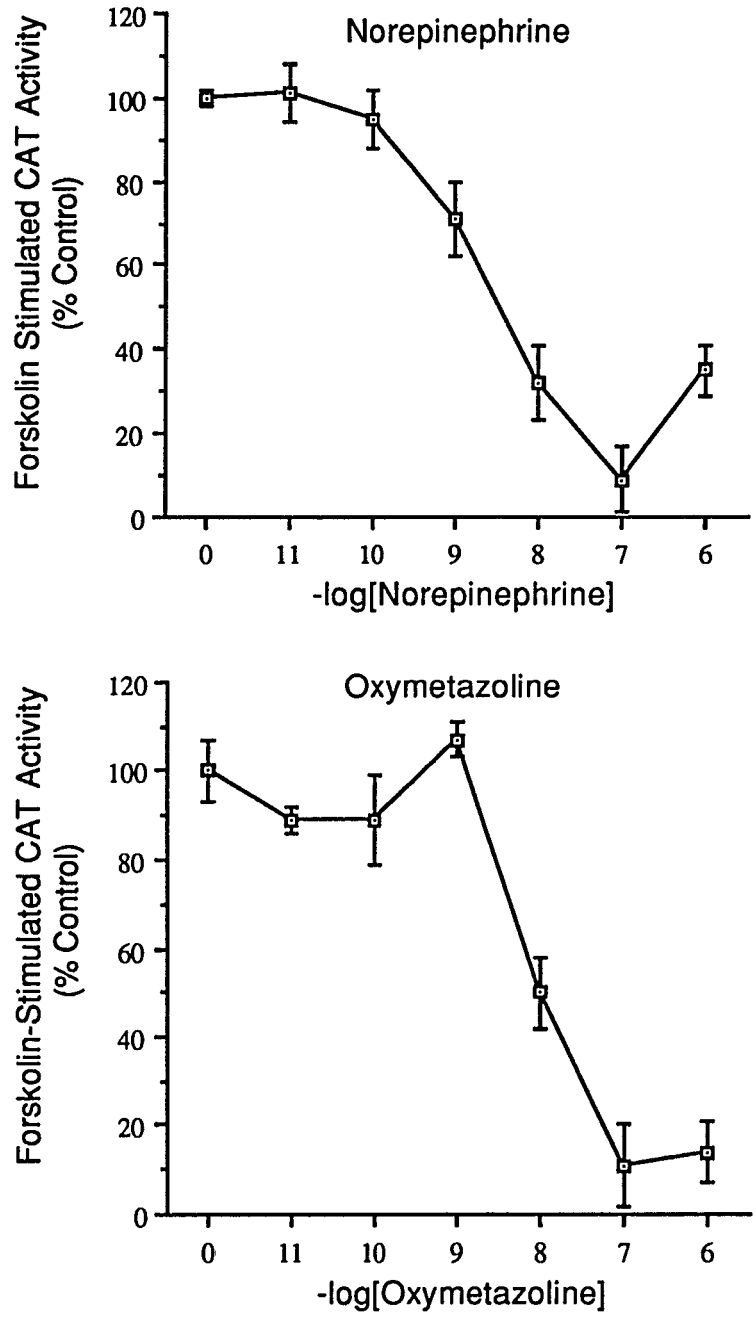


Figure 18. Effect of agonists norepinephrine and oxymetazoline on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C4 DNA.

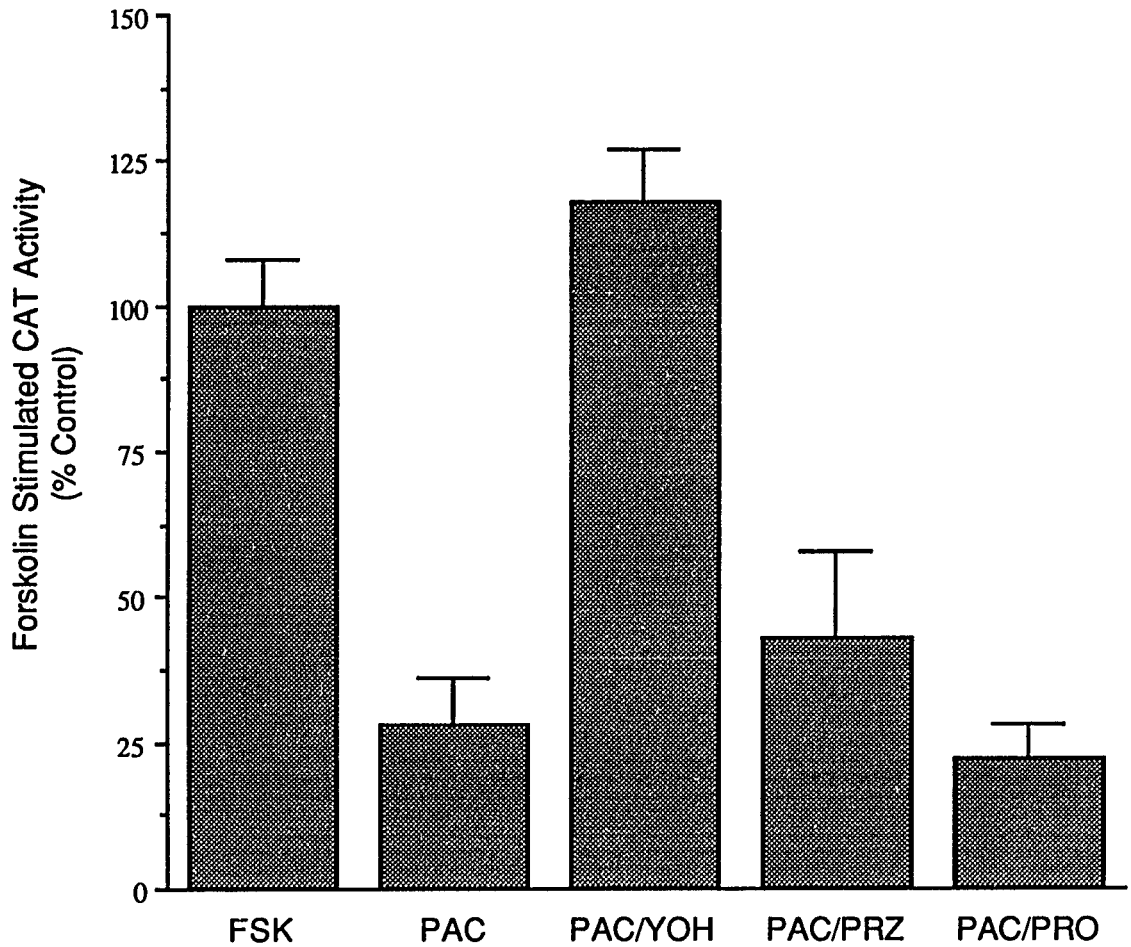


Figure 19. Effect of adrenergic antagonists yohimbine (YOH), prazosin (PRZ) and propranolol (PRO) on the inhibition of CAT activity induced by p-aminoclonidine (100 nM) in α_2-C4 transfected JEG-3 cells. Forskolin and antagonists were added at a 1 μM final concentration. Each bar represents the mean ± S.E. from three individually-transfected plates.

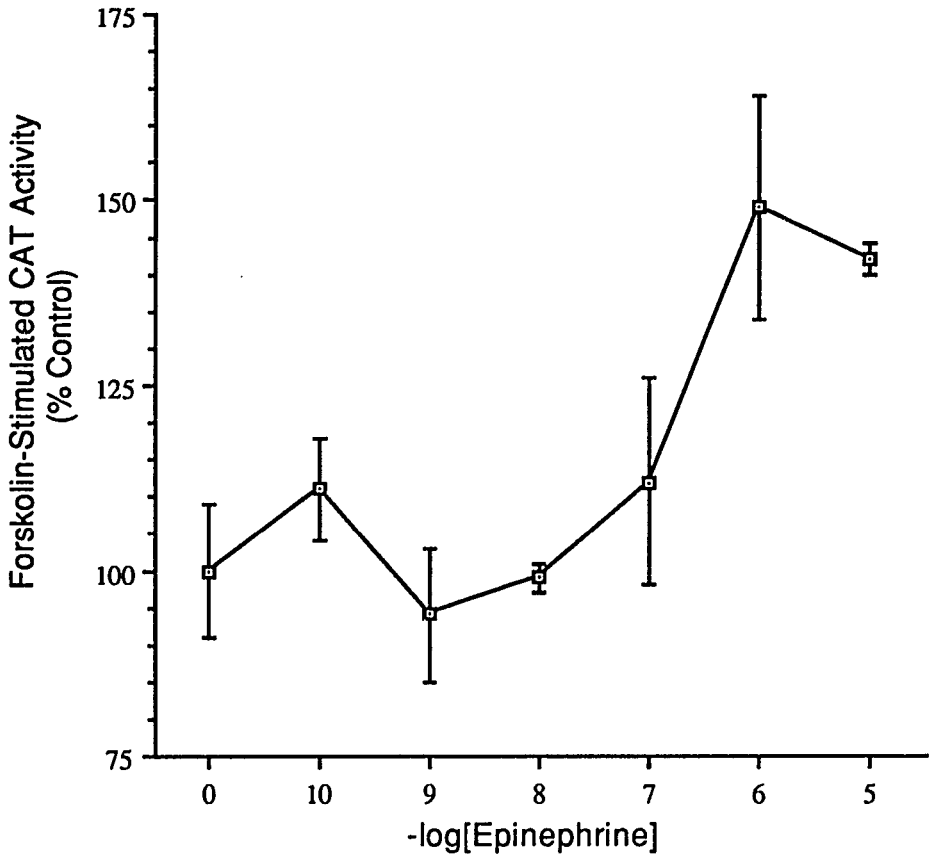


Figure 20. Effect of epinephrine on forskolin-stimulated CAT activity in JEG-3 cells transfected with the α_2-C4 adrenergic receptor coding plasmid and pre-treated for 18 hours with 100 ng/ml pertussis toxin prior to drug incubation. Each point represents the mean $\pm$ S.E.M. for three plates of transfected cells.

3.2.3 α_2-C2

In a similar manner, we examined functional responses in JEG-3 cells transfected with the reporter plasmid pCRE(+) and pBCα_2-C2. Expression of this receptor subtype in JEG-3 cells was intermediate to that of the α_2-C4 (low) and α_2-C10 (high). In six separate experiments, expression of the α_2-C2 averaged ~0.75 pmol/mg protein. Antibody labelling of these transiently-transfected cells revealed that about 0.5-1% of the cells became transfected and expressed the receptor protein. This labelling was found mainly on the plasma membrane, with little distinct labelling of the nucleus or other intracellular organelles.

In contrast to both the α_2-C4 and α_2-C10 receptors, agonists elicited a purely stimulatory effect on forskolin-stimulated CAT activity in α_2-C2 transfected JEG-3 cells. Figure 21 shows that the agonists epinephrine and medetomidine produced dose-dependent increases in CAT activity that were similar to the maximal stimulation observed for cells transfected with the α_2-C10 (~1.4 fold). Medetomidine was again more potent than epinephrine at this subtype. p-Aminoclonidine (figure 22) was approximately equipotent with epinephrine at this subtype, while UK-14304 displayed very low potency (EC_{50} ~500 nM). Norepinephrine (figure 23) was about 5-fold less potent than epinephrine, and oxymetazoline, which typically exhibits very low radioligand binding affinity for this subtype, possessed low potency but rather good efficacy in α_2-C2 transfected cells. p-Aminoclonidine (100 nM) elicited a 70-80% potentiation of forskolin-stimulated CAT activity (Figure 24), which could be specifically blocked by yohimbine, but not by either prazosin or propranolol, demonstrating that this response is clearly mediated by the

transfected α_2-C2 receptor subtype. Pertussis toxin had little effect on the epinephrine potentiation of forskolin-stimulated CAT activity in α_2-C2 transfected cells (figure 25).

Since the α_2-C2 receptor subtype stimulates, rather than inhibits CAT activity in this system, forskolin was not required to measure a functional response. We then determined if we could use this transient expression system to antagonize a functional agonist effect using receptor antagonist. It then might be feasible to measure the dissociation constant (K_B) for a receptor antagonist. To test this hypothesis, JEG-3 cells were transiently-transfected with the reporter plasmid and plasmid encoding the α_2-C2 receptor subtype. Medetomidine dose-response curves were then performed in the presence or absence of a fixed concentration of yohimbine. Since the K_D value for yohimbine at the α_2-C2 receptor is well established (Bylund *et al.*, 1992), a concentration of 10 nM, which corresponds to this Kd value was initially chosen. This dose produced no observable shift in the medetomidine dose response in these cells. Subsequently, we examined the effect of 100 nM yohimbine. This concentration of yohimbine produced a 5-fold shift in the medetomidine dose response (figure 26). In control cells the EC_{50} for stimulation was 4 nM, whereas in yohimbine-treated cells the respective EC_{50} value was 20 nM. Using the equation (Tallarida *et al.*, 1988):

$$\frac{[A']}{[A]} - 1 = \frac{[B]}{K_{DB}}$$

where [A] = the EC_{50} value in the absence of antagonist.

[A'] = the EC_{50} value in the presence of competitive antagonist.

[B] = the concentration of competitive antagonist.

K_{DB} = the dissociation constant for the antagonist

a value of 25 nM was obtained for the antagonist dissociation constant for yohimbine. While this value is higher than both the Kd value obtained in direct binding studies (chapter 4) and the K_i values obtained for the α_2-C4 in other cell systems (Bylund *et al.*, 1992), this result clearly demonstrates our ability to measure antagonism of a functional response in an intact cell system.

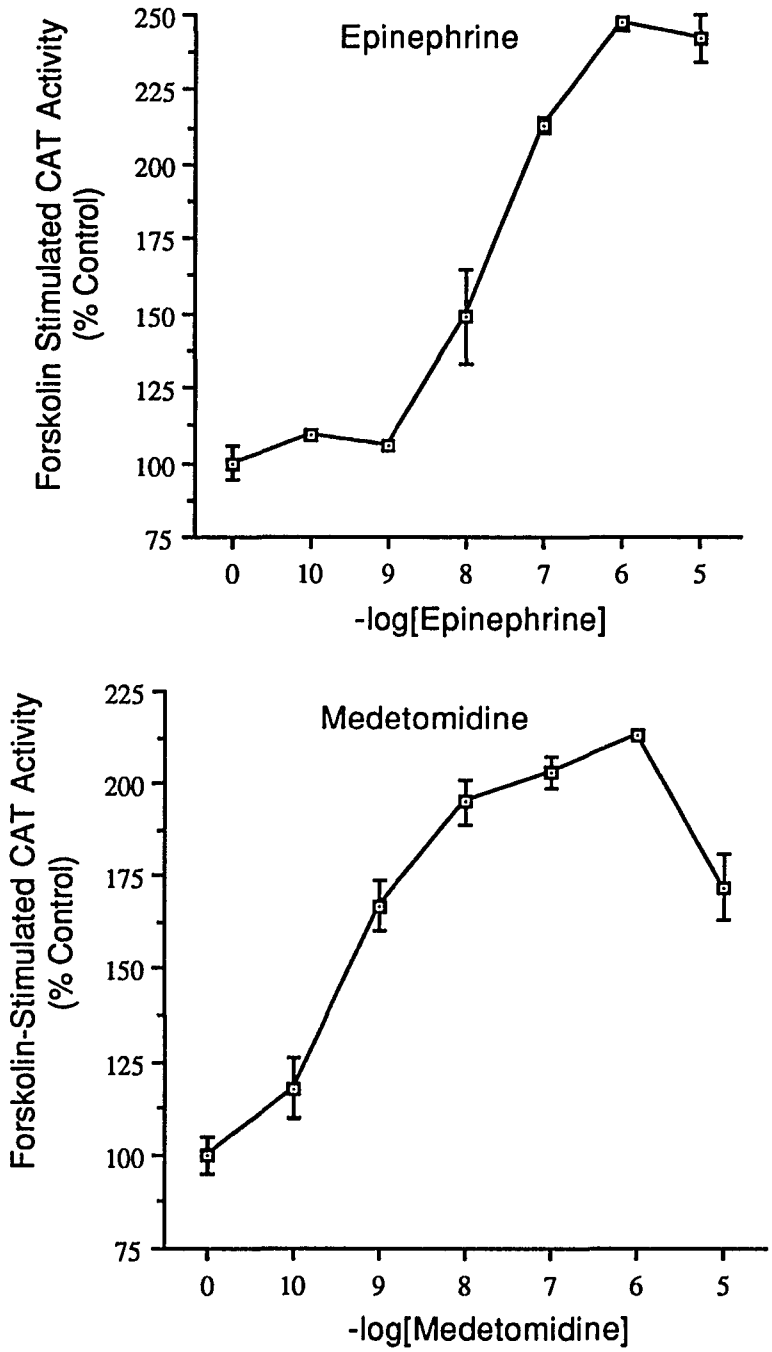


Figure 21. Effect of α_2-adrenergic receptor agonists epinephrine and medetomidine on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid pCRE(+) and pBCα_2-C2 DNA. Each point represents the mean $\pm$ S.E. for three individually-transfected plates.

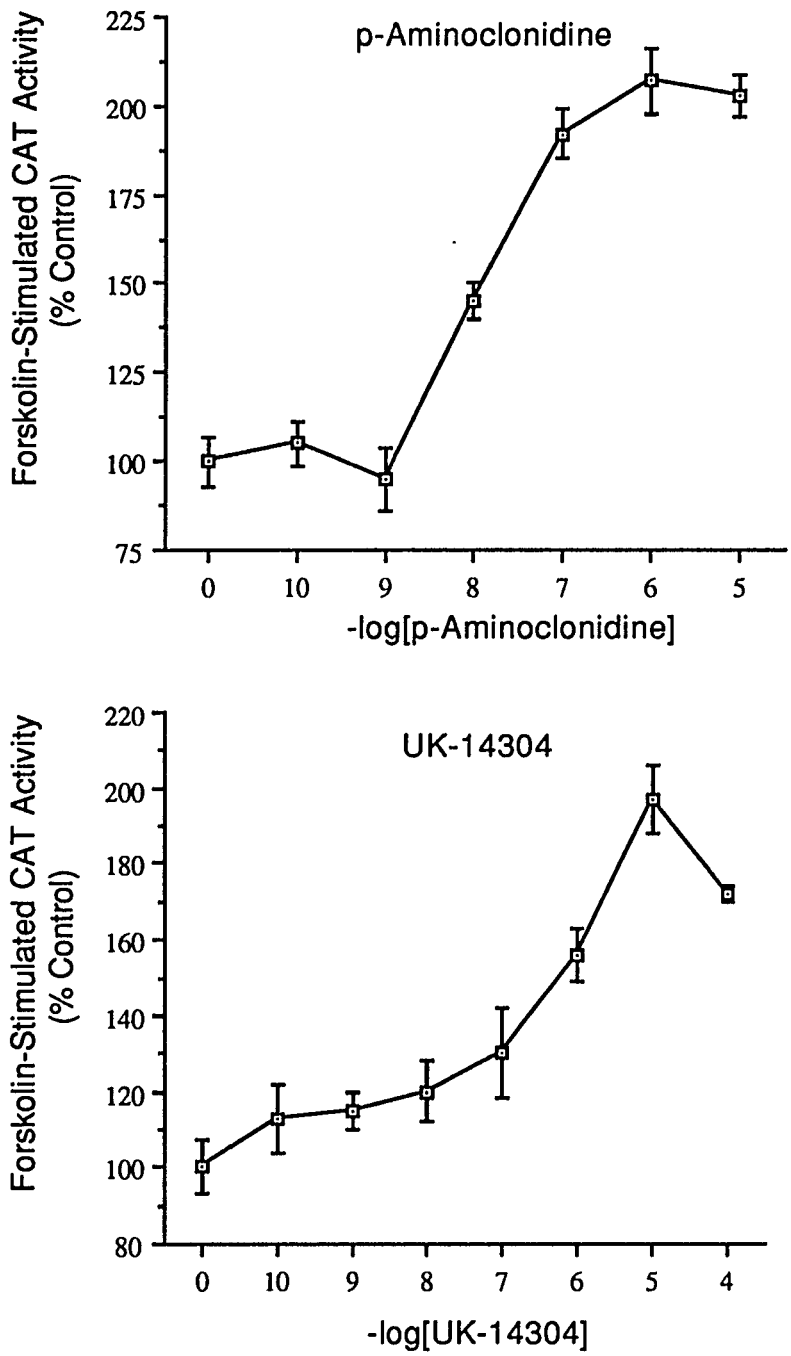


Figure 22. Effect of α_2-adrenergic receptor agonists p-aminoclonidine and UK-14304 on forskolin-stimulated CAT activity in α_2-C2 transfected JEG-3 cells. Each point represents the mean + S.E. from three individually-transfected plates.

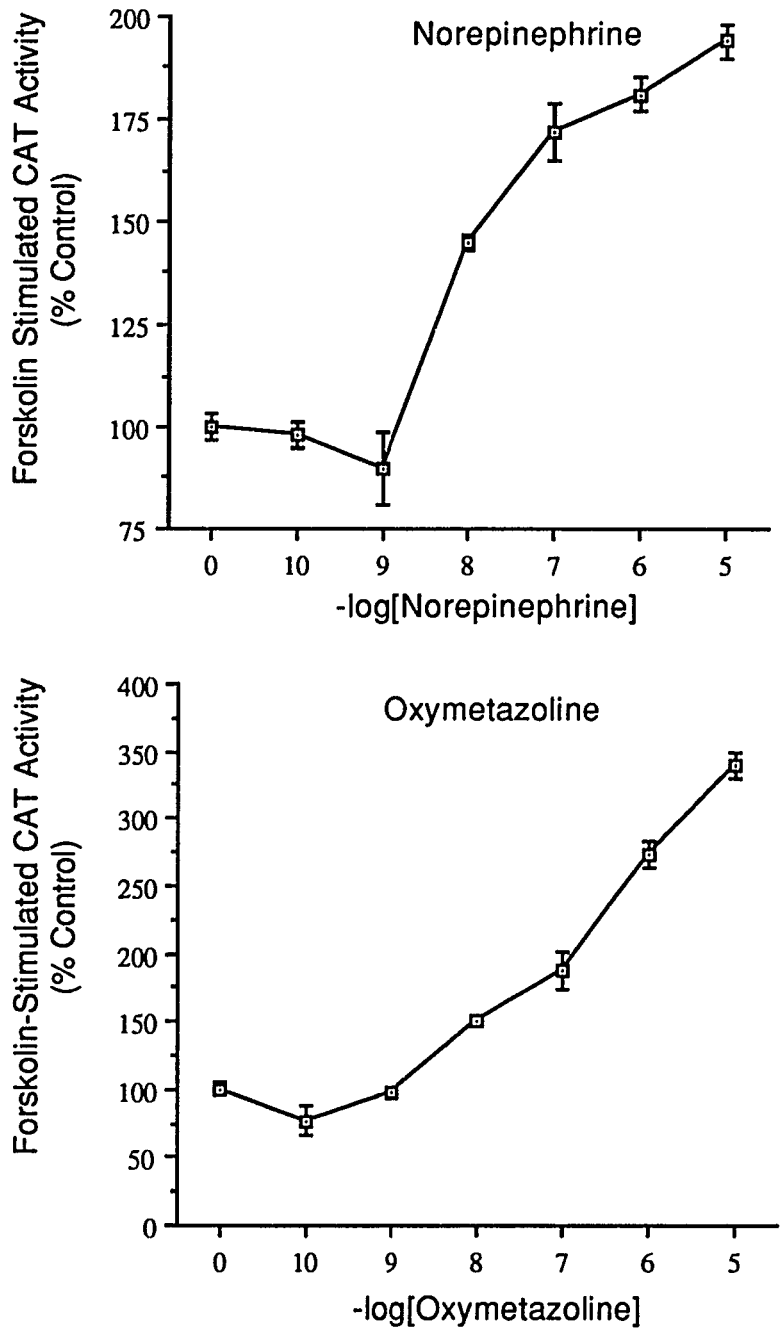


Figure 23. Effect of agonists norepinephrine and oxymetazoline on forskolin-stimulated CAT activity in α_2-C2 transfected JEG-3 cells. Each point represents the mean ± S.E. from three individually-transfected plates.

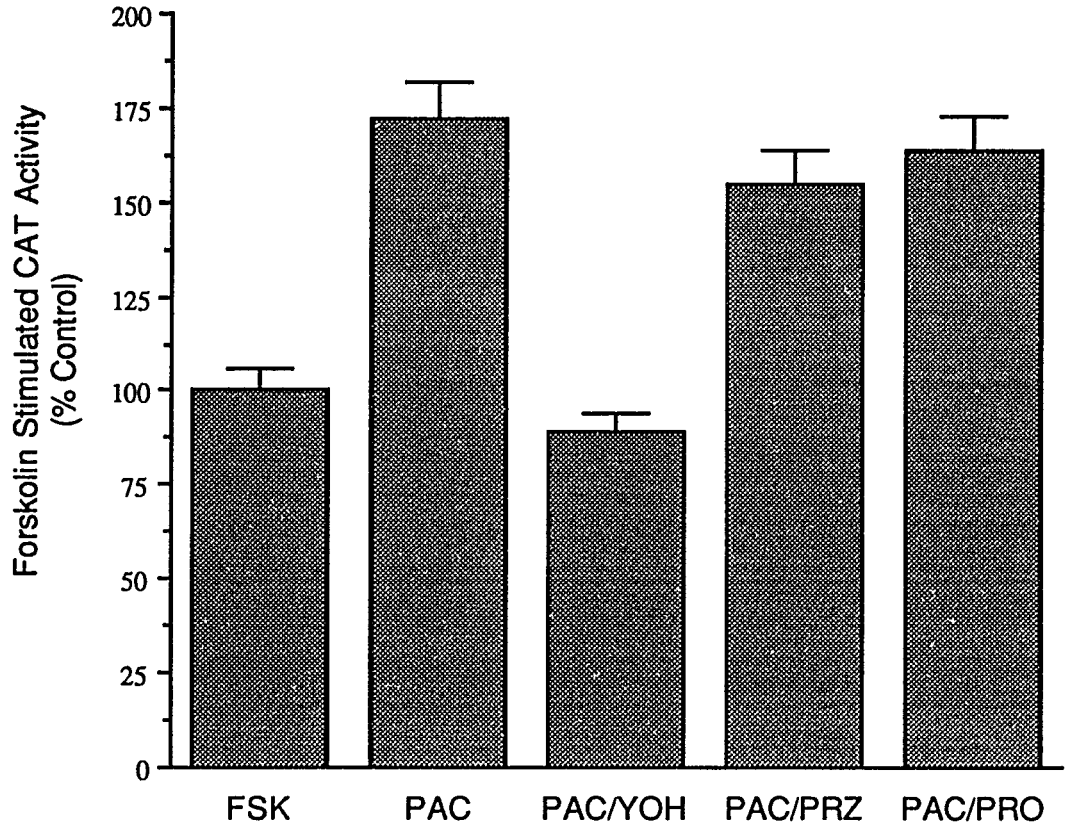


Figure 24. Effect of adrenergic antagonists yohimbine (YOH), prazosin (PRZ) and propranolol (PRO) on the potentiation of CAT activity induced by 100 nM p-aminoclonidine in α_2-C2 transfected JEG-3 cells. Forskolin and antagonists were added at 1 μM final concentration.

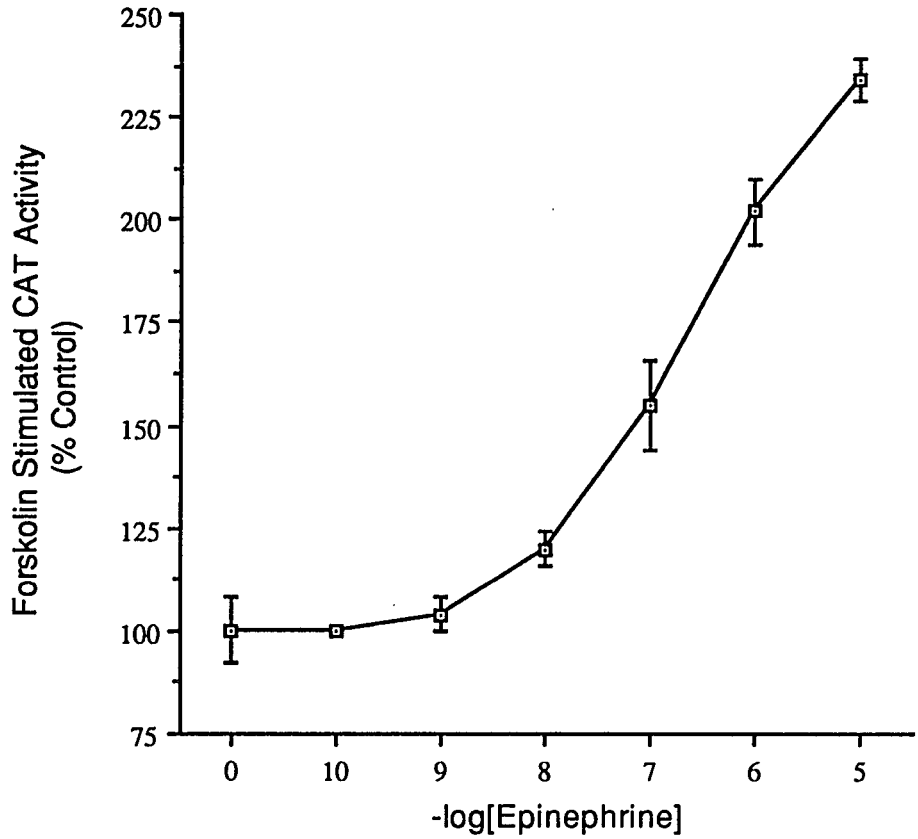


Figure 25. Effect of epinephrine on forskolin-stimulated CAT activity in JEG-3 cells transfected with the reporter plasmid and the α_2-C2 receptor coding plasmid and pre-treated for 18 hours with 100 ng/ml pertussis toxin prior to drug incubation. Each point represents the mean $\pm$ S.E. of three individually-transfected plates.

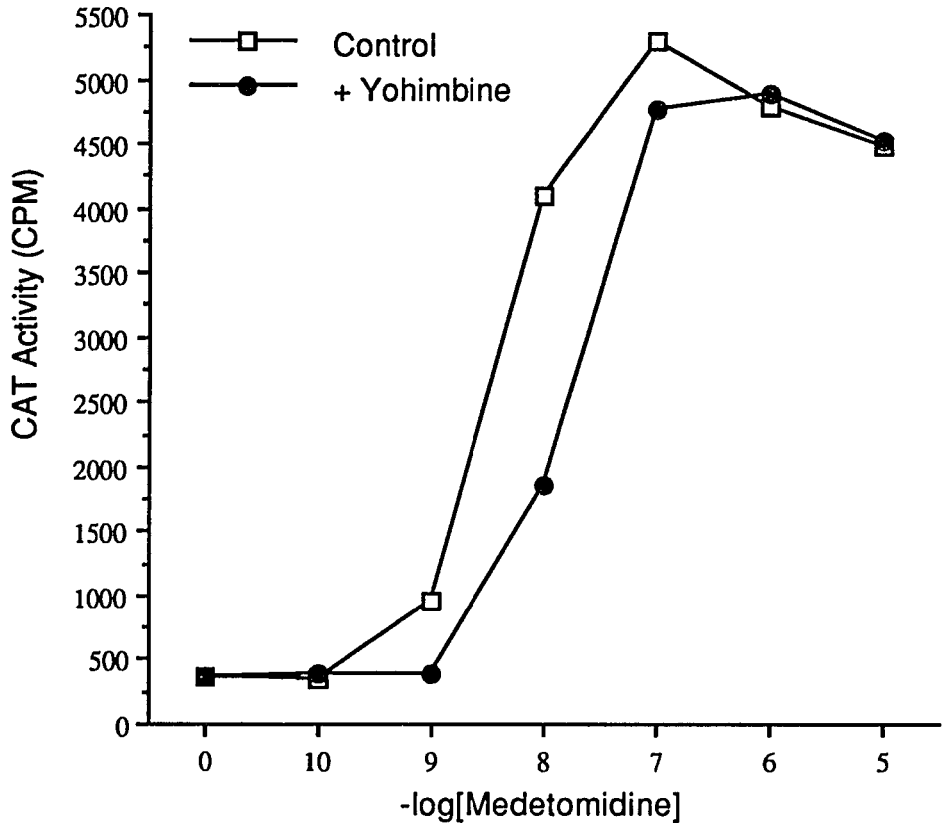


Figure 26. Effect of 100 nM yohimbine on medetomidine stimulation of CAT activity in JEG-3 cells transfected with the α_2-C2 adrenergic receptor subtype. Each point represents a single plate of transfected cells.

3.2.4 α_2-F

Alpha-2 adrenergic receptors have also been defined in non-mammalian species as well. Receptors with an α_2 adrenergic receptor pharmacology have been characterized in the skin of lower vertebrates, where they appear to mediate pigment cell (melanophore) aggregation, leading to a color change (Berthelsen and Pettinger, 1977). Recently, a fish-skin α_2 receptor has been cloned from a cuckoo wrasse (*Labrus ossifagus*) genomic library (Svensson *et al.*, 1993b). This fish receptor, termed α_2-F, is the first non-mammalian α_2 receptor to be cloned and sequenced. This receptor is of similar length to the human α_2 receptors and possesses 57% overall homology to the human α_2–C4 receptor subtype, 82% in the transmembrane domains. Like most adrenergic receptors, this fish α_2-receptor does not contain introns and possesses many of the conserved structural features involved in ligand binding and effector coupling. Consensus sequences for regulatory transcription factors are also present in the 5'-untranslated region, suggesting that expression of this fish α_2 gene may be regulated by both cAMP and steroid hormones. Although structurally very similar to the human α_2 receptors, the α_2-F does exhibit a unique structural feature. Whereas all other α_2 cloned receptors contain a sequence of three phenylalanines (FFF) in their seventh putative transmembrane regions (Pepperl and Regan, 1993a), the fish α_2 receptor possesses only two. This missing phenylalanine residue, homologous to phe-412 in the human α_2–C10 receptor, has recently been implicated in conferring antagonist binding specificity to the β_2 adrenergic receptor (Suryanarayana *et al.*, 1991).

In order to characterize the pharmacology of the α_2-F, we examined the effect of several α_2 receptor agonists on forskolin-stimulated CAT activity in transiently-transfected JEG-3 cells. We might then determine which of the human α_2 receptors the α_2-F most resembles functionally. Based on its structural homology with the α_2-C4, we would predict that it would function most like this receptor subtype. To test this hypothesis, the α_2-F was transiently expressed in JEG-3 cells in the pBC12BI expression vector, and agonist dose-response curves were performed similar to the other receptors. Figure 27 shows the effect of agonists epinephrine and UK-14304 (top) and medetomidine and oxymetazoline (bottom) in α_2-F transfected JEG-3 cells. Each agonist elicited purely dose-dependent inhibition of forskolin-stimulated CAT activity, similar to the α_2-C4 receptor subtype. Medetomidine and oxymetazoline were the most potent agonists at this subtype with EC_{50s} of 0.16 and 0.21 nM, respectively. Epinephrine and UK-14304 were also agonists at the α_2-F expressed in JEG-3 cells, and were substantially less potent, with EC_{50} values of 4.4 and 3.1 nM, respectively. p-Aminoclonidine was also an agonist at this subtype with a potency about equal to that of epinephrine (table 3). These experiments have shown that the fish α_2 adrenergic receptor may be transiently expressed in JEG-3 cells and that functionally, it resembles the α_2-C4 receptor subtype. Moreover, in contrast to the fish melanophore system, UK-14304 is a potent agonist in JEG-3 cells expressing the α_2-F, suggesting that the effects of this compound may depend highly on the cell type in which it is examined.

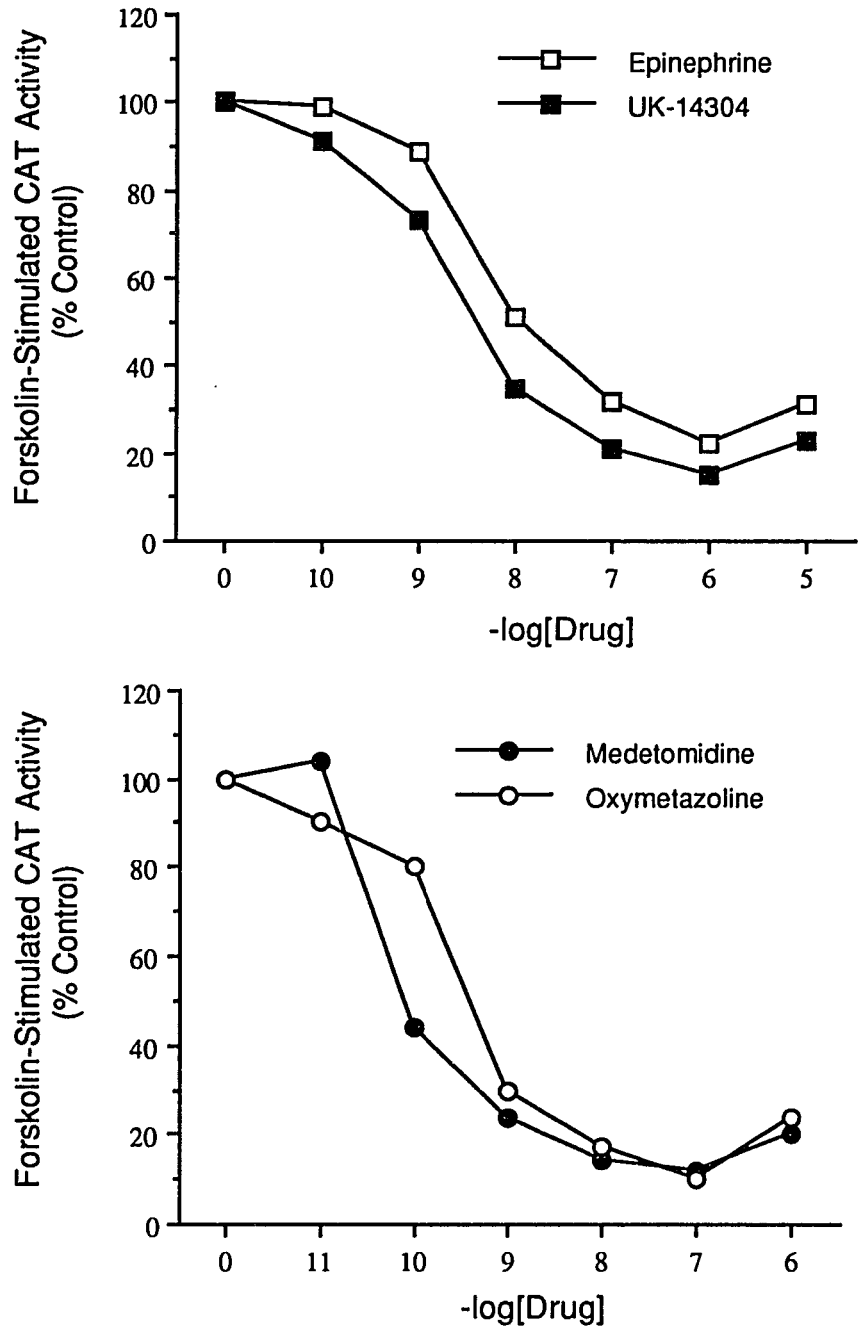


Figure 27. Effect of α_2 receptor agonists epinephrine and UK-14304 (top), and medetomidine and oxymetazoline (bottom) in JEG-3 cells transfected with reporter plasmid pCRE(+) and plasmid encoding the fish α_2 adrenergic receptor. Each point represents the mean of two individually-transfected plates.

TABLE 2

AGONIST POTENCIES FOR FORSKOLIN-STIMULATED CAT EXPRESSION IN JEG-3 CELLS TRANSFECTED WITH α_2-ADRENERGIC RECEPTOR SUBTYPES*

	EC_{50} (nM)			
COMPOUND	α_2-C2	α_2-C4	α_2-C10	α_2-F
epinephrine	40	20	0.2	4.4
norepinephrine	200	3.2	3.2	ND
p-aminoclonidine	16	3.2	0.06	4.0
medetomidine	0.6	0.2	0.2	0.16
UK 14304	500	2.0	6.3	3.1
oxymetazoline	6.3	10	ND	0.21

*Potencies for either the potentiation (α_2-C2) or the inhibition (α_2-C4, α_2-C10 and α_2-F) of forskolin-stimulated CAT expression were determined from the data shown. ND = not determined.

3.3 Discussion

In this study we have used transient expression in human choriocarcinoma (JEG-3) cells to compare the pharmacology of the cloned human and fish α_2 adrenergic receptor subtypes. Rather than simply compare radioligand binding properties, we sought to determine the effect of various α_2 adrenergic receptor agonists on forskolin-stimulated CAT activity in cells transfected with either the α_2-C2, α_2-C4, α_2-C10 or α_2-F adrenergic receptor subtypes. The purpose of the present studies was two-fold. First, we wished to define the agonist pharmacology in the JEG-3 cell system for future mutagenesesis studies, and second, to compare the various receptor subtypes with respect to 2nd messenger coupling.

Although closely related in structure (Lomasney *et al.*, 1990), binding properties (Bylund *et al.*, 1992), and coupling to G-proteins (Eason *et al.*, 1992), (Jansson *et al.*, 1994), activation of individual α_2 adrenergic receptor subtypes can, nevertheless, result in the production of unique intracellular responses. Thus, agonists produced a biphasic response in α_2-C10 transfected cells, a purely inhibitory response in α_2-C4 and α_2-F transfected cells, and a purely stimulatory response in cells transfected with the α_2-C2 receptor subtype. In each case, inhibition of forskolin-stimulated CAT activity could be ablated by pre-treatment of the cells with pertussis toxin, whereas the potentiation of CAT activity observed with the α_2-C10 and α_2-C2 receptors was insensitive to the toxin. These results imply then, that the α_2 adrenergic receptors can couple to two independent signal transduction pathways in the JEG-3 cell system.

With respect to the α_2-C10 adrenergic receptor, we observed a biphasic effect on forskolin-stimulated CAT activity in the JEG-3 cell system. Low doses of agonist inhibited forskolin-stimulated CAT activity, with maximal inhibition of ~60% in each case (figures 10-12). This inhibition was followed at higher doses by a dose-dependent reversal (PAC, UK-14304, and medetomidine) or potentiation (epinephrine, norepinephrine). Based on our results with pertussis toxin (figure 15), these effects appear to result from activation of distinct G-proteins within the host cell. This biphasic coupling phenomena has previously been observed in a number of cell systems with the α_2-C10 receptor, in particular stably-transfected CHO cells (Fraser *et al.*, 1991) and transfected mouse S115 mammary tumor cells (Jansson *et al.*, 1994). This agonist-induced stimulation of cAMP appears to involve direct coupling of the α_2 receptors with the stimulatory G-protein G_s (Eason et al., 1992).

With respect to agonist potencies, our results compare favorably with measurements of adenylyl cyclase activity in stably transfected CHO cells. For example, the EC_{50} of epinephrine for decreasing cAMP at the α_2-C10 in stably transfected CHO cells is 3 nM, whereas the EC_{50} for increasing cAMP is in the micromolar range (Eason *et al.*, 1992). For epinephrine, the EC_{50} for inhibition of forskolin-stimulated CAT activity in JEG-3 cells was 0.2 nM, and the EC_{50} for the potentiation of CAT activity was also in the micromolar range. The present results also agree with measurements of adenylyl cyclase activity in stably transfected CHO cells (Eason *et al.*, 1992). Thus, the EC_{50} of UK-14304 for inhibiting adenylyl cyclase at the α_2-C10 was 90 nM while its EC_{50} for the inhibition of forskolin-stimulated CAT activity in JEG-3 cells was 6 nM.

For both the stimulation of adenylyl cyclase activity in CHO cells, and the potentiation of CAT activity in JEG-3 cells, the EC_{50} of UK-14304 at the α_2-C10 was in the μM range.

Our results with the α_2-C10 also suggest that, for a given receptor subtype, different agonists have different abilities to influence either the inhibition or the potentiation of CAT activity. Thus, both epinephrine and PAC produced a similar degree of inhibition but differed in their ability to potentiate forskolin-stimulated CAT activity. This has been confirmed with other agonists: norepinephrine will potentiate CAT activity 1.5 fold, whereas UK-14304 and medetomidine at high doses will only return CAT activity to the control level. This difference in the ability of agonists to maximally potentiate CAT activity at the α_2-C10 suggests a possible difference in the way in which catecholamines (epinephrine and norepinephrine) and imidazolines (PAC, UK 14304 and medetomidine) interact with the receptor. Thus, these two classes of α_2-adrenergic receptor agonists may share overlapping but nonidentical binding sites; the consequence of which might be that the binding of catecholamines to the receptor produces a slightly different conformational change that allows more effective coupling of the receptor to the cAMP-potentiating pathway. Similar results were only recently obtained for the α_2-C10 following stable expression in CHO cells. In these studies, epinephrine and norepinephrine were again the most efficacious in terms of stimulating cAMP production, while oxymetazoline had little effect at this receptor subtype (Eason *et al.*, 1994).

In contrast to the biphasic pattern observed with the α_2-C10 receptor, agonists elicited only inhibition of forskolin-stimulated CAT activity in α_2-C4 transfected cells. Even oxymetazoline, ineffective at the α_2-C10 receptor

subtype, potently inhibited forskolin-stimulated CAT expression in cells expressing the α_2-C4 receptor subtype. A similar pattern was obtained in cells transfected with the α_2-F, suggesting that functionally, this fish receptor most closely resembles the α_2-C4 receptor subtype. Unlike the fish melanophore system however, UK-14304 was a rather potent agonist at the α_2-F, with an EC_{50} of 3.1 nM. For the α_2-F, medetomidine was the most potent agonist at this subtype, followed by oxymetazoline, UK-14304, PAC and epinephrine.

Unlike the α_2-C10, we did not observe agonist-induced potentiation of forskolin-stimulated CAT activity in cells transfected with the α_2-C4 receptor subtype. Even following pertussis toxin treatment of these cells, epinephrine produced only a very slight potentiation of forskolin-stimulated CAT activity at the highest doses. This result was in stark contrast to the robust (~280%) potentiation of forskolin-stimulated CAT activity observed in pertussis toxin-treated, α_2-C10 transfected cells (figure 15). At least for the α_2-C10, it has been clearly shown that this potentiation of cAMP formation, and of adenylyl cyclase activity, is a function of its level of expression (Fraser *et al.*, 1989). In stably transfected CHO cells, 'low' levels of expression of the α_2-C10 (0.5-1.0 pmol/mg protein) yielded primarily inhibition and high levels of expression (>1.0 pmol/mg) yielded a biphasic dose-response curve, with low concentrations of epinephrine inhibiting cAMP formation and high concentrations stimulating formation. While it is still possible that the functional differences we have observed between the α_2 receptor subtypes may simply reflect their level of expression, this is not likely the case. Although overall expression levels varied only from ~0.4 pmoles/mg for the α_2-C4 to

~1.2 pmoles/mg for the α_2-C10, actual levels of expression in the transfected cell population are probably much higher. This would suggest then, that the receptors are in relatively large excess compared to other signaling molecules. In this scenario, receptor-effector interactions would be determined based on the receptor's intrinsic affinity for selected G-proteins. Thus, we would conclude that the α_2-C4 receptor does not significantly interact with the stimulatory G-protein(s) in these cells.

For the α_2-C2 adrenergic receptor, only stimulatory responses to agonist were observed. In each case, agonists produced 100-150% potentiation of forskolin-stimulated CAT activity, except for oxymetazoline, which potentiated CAT expression ~250%. Inhibitory activity as a result of receptor activation has not been observed in any of our experiments with the α_2-C2. While it has been shown, at least for the α_2-C10, that receptor expression levels can affect the coupling to the cAMP-stimulatory pathway (Eason *et al.*, 1992), receptor expression levels have not been reported as a factor affecting coupling of α_2-adrenergic receptors to inhibition of adenylyl cyclase or cAMP production (Fraser *et al.*, 1989). In contrast to the α_2-C10, where the potentiation of CAT activity by agonists is in the μM range, for the α_2-C2 the EC_{50}'s are in the nM range. This could indicate that, at least for α_2-C2, the ability to stimulate cAMP formation is physiologically significant. If the results of our experiments reflect adenylyl cyclase activity, then the α_2-C2, at least in JEG-3 cells would appear to couple predominantly to the stimulation of cyclase.

While it is clear that the α_2-C10 and α_2-C4 can functionally couple to distinct G-protein α subunits (Kurose *et al.*, 1991), coupling of the α_2-C2 to

selected G-proteins has not been extensively examined. Only recently it was demonstrated that the JEG-3 cells express $G_{\alpha s}$, $G_{\alpha i1}$ and $G_{\alpha i3}$, but appear to lack the $G_{\alpha i2}$ subunit (Montmayeur *et al.,* 1993). This apparent lack of $G_{\alpha i2}$ in these cells offers a potential explanation for our inability to observe agonist-mediated inhibition of forskolin-stimulated CAT activity in cells expressing the α_2-C2 receptor subtype. If the α_2-C2 receptor subtype were to couple exclusively to $G_{\alpha i2}$, then in cells lacking this G protein one would not expect to observe agonist-mediated inhibition of cAMP production, and hence cAMP-dependent CAT activity. One possible means for testing this hypothesis would involve a three-way transfection of reporter plasmid, α_2-C2 receptor coding plasmid, as well as plasmid encoding the α subunit of G_{i2}. If agonist-induced inhibition of forskolin-stimulated CAT activity were observed following co-transfection of the α_2-C2 receptor and $G_{\alpha i2}$, then we could conclude that the α_2-C2 couples solely to this inhibitory G-protein α subunit.

In terms of studying receptor function, this transient system, with its use of a CRE-CAT reporter gene, will never entirely replace stably-expressing cell lines for studying receptor-effector interactions. However, this system does provide an easier, more rapid alternative to stable gene expression. More importantly, this transient system appears to reveal distinct functional differences which may go unnoticed in more traditional, stable expression systems. It is certainly possible that functional differences between the α_2-receptor subtypes might not be appreciated by biochemical determinations that are either too close in the pathway (e.g. GTPase or adenylyl cyclase) or too close in time with respect to receptor activation. A possible advantage of the present system is that it is more downstream and it may reflect a

summation of more than one signaling pathway. It is significant that in an otherwise identical system, the activation of closely related subtypes can produce unique intracellular responses.

Finally, these experiments clearly reflect the potential regulation of gene expression by α_2 adrenergic receptors. As described in chapter 1, α_2 receptors are present in a wide variety of tissues, each of which expresses a distinct set of genes. While the short-term effects of receptor activation (ie inhibition of adenylyl cyclase, G-protein activation), are becoming evident, the long-term consequences of α_2 adrenergic activation are still unclear. With the discovery of both positive and negative regulators of gene expression (Meyer and Habener, 1993), the potential for distinct signaling events by closely-related receptor subtypes becomes even greater.

CHAPTER 4

STABLE EXPRESSION OF α_2 ADRENERGIC RECEPTOR SUBTYPES IN JEG-3 CELLS

4.1 Introduction

Clearly, the JEG-3 cells provide a suitable host for functional expression of α_2 adrenergic receptors. Using this transient system, we can rapidly examine both the pharmacology and effector coupling for the various α_2-adrenergic receptor subtypes. More importantly, this approach appears to reveal distinct differences in receptor-effector coupling, not previously observed in other systems (Eason *et al.*, 1992). While both the α_2-C4 and α_2-C10 receptors appear to couple to inhibition of forskolin-stimulated reporter gene activity, the α_2-C2 subtype exhibited a purely stimulatory response to agonist. While it has been previously shown that the α_2-C2 subtype can couple to inhibition of adenylyl cyclase in stably-transfected CHO cells (Eason *et al.*, 1992), this may not necessarily be true in JEG-3 cells. Recently, it was shown that the α_2-C2 subtype could couple more effectively to stimulation of cAMP production in stably-transfected mouse mammary tumor cells than either the α_2-C4 or α_2-C10 receptor subtypes (Jannson *et al.*, 1993). These results have shown that in certain systems, the α_2-C2 can effectively couple to stimulation of adenylyl cyclase.

Although the reporter plasmid used for these studies is ultimately driven by cAMP, we do not know how closely our CAT measurements reflect actual cAMP concentrations in these cells. One reason for this is that direct measurements of cAMP in this transient expression system are of little value.

Typically, only a small fraction of the cells are actually transfected (<10%). Thus, when stimulated by forskolin, the concentration of cAMP will increase in all the cells but will only be decreased in those cells expressing α_2-receptors. The present system works because the same population of cells takes up both the receptor plasmid and reporter plasmid.

Thus, in order to determine if CAT activity truly reflects cAMP production, stably-transfected JEG-3 cell lines expressing the α_2 adrenergic receptor subtypes were prepared. We subsequently characterized the pharmacology and 2nd messenger coupling of the α_2 adrenergic receptor subtypes stably expressed in JEG-3 cells.

4.2 Materials and Methods

4.2.1 Selection of Stably-Expressing JEG-3 Cell Lines

Plasmid pRX-Neo was obtained from the laboratory of Dr. Roger Miesfeld. This plasmid constitutively expresses the gene for neomycin resistance downstream of the SV40 early promoter. The α_2 adrenergic receptors were expressed in pBC12BI as previously described (Chap. 2). JEG-3 cells were co-transfected with pRX-NEO and either α_2-C2, α_2-C4 or α_2-C10 plasmid DNAs (1:10 ratio), using the calcium phosphate technique (Appendix C). Cells were allowed to grow for 36-40 hours following transfection and were then maintained in DMEM containing 0.5 mg/ml Geneticin (G418). Antibiotic resistant foci were transferred to new wells and propagated in DMEM + 0.5 mg/ml G418. Clones were screened for α_2 adrenergic receptor expression by single-point radioligand binding analysis using 10-12 nM [^{3}H]rauwolscine. Clones exhibiting specific binding above 0.2-0.3 pmol/mg were maintained for further analysis.

4.2.2 Membrane Preparation and Radioligand Binding Studies

Saturation and competition radioligand binding studies (Regan *et al.*, 1988) were used to characterize the pharmacology of α_2 adrenergic receptors expressed in the JEG-3 cells. Membranes were first prepared by rinsing confluent plates of JEG-3 cells twice with phosphate buffered saline, and scraping the cells into TME buffer (50 mM Tris-HCl/10 mM $MgCl_2$/1 mM EDTA). Lysates were prepared using a Brinkman homogenizer, and

membranes were pelleted at 48,000xg for 20 minutes at 4°C and resuspended in TME buffer. Membranes were either used immediately for saturation analysis or frozen at -80°C for subsequent use. Protein concentration was determined using a Bio-Rad protein assay reagent with a standard curve of 0-15 μg BSA.

For saturation studies, 5-25 μg of membrane protein were used in a final volume of 500 μl. [^{3}H]Rauwolscine was used at 0.05-12 nM and non-specific binding was determined at 0.5, 2 and 8 nM concentrations, and extrapolated for the remaining concentrations. Incubations were for 1 hour at 25°C and were terminated by rapid filtration onto untreated Whatman GF/B filters, followed by four washes with sodium-potassium phosphate buffer (40 mM Na_2HPO_4/10 mM KH_2PO_4, pH 7.4). B_{MAX} and K_D values for stably-expressed α_2 receptors were calculated using the INPLOT4 program fit to a one-site model. For competition radioligand binding studies, 25-100 μg of membrane protein were used and [^{3}H]rauwolscine was used at 0.5-1 nM final concentration. K_i values for inhibition of [^{3}H]rauwolscine binding were determined according to Cheng and Prusoff (1973).

4.2.3 Drug incubations and [^{3}H]cAMP Assay

Control and stably-transfected JEG-3 cell lines were assayed for cAMP according to a modified version of Gilman (1970). Cells from a single 10 cm dish were trypsinized and plated in 24-well format two days prior to assay. After 24 hours, the media was removed and replaced with serum-free media lacking G418. Drug solutions were prepared as 5x stock solutions in DMEM containing 0.1 mg/ml isobutylmethylxanthine (IBMX). Cells were placed at 37°C and were rinsed once with serum-free media and fed 400 µl of DMEM containing IBMX. One hundred microliters of drug dilution were added and incubated with the cells for either three or 15 minutes as described in figure legends. Following the drug incubation, the media was aspirated and the reaction terminated by addition of 150µl of 50 mM tris/4 mM EDTA. Cells were placed on ice, scraped into buffer and boiled for 5-10 minutes. Samples were typically frozen at -20°C prior to assay.

cAMP assays (Appendix D) included 50 µl of cytosol, 50 µl of [^{3}H]cAMP and 100 µl of protein kinase A solution. Samples were incubated for two hours on ice, and the unbound cAMP precipitated by addition of 2%BSA/charcoal solution. Samples were centrifuged briefly and 200 µl of supernatant were transferred to scintillation vials for counting. [^{3}H]cAMP in the supernatant was determined by comparison to a standard curve from 0.125-64 pmoles cAMP.

4.3 Results

4.3.1 Stable Cell Selection and Radioligand Binding Experiments

Since JEG-3 cells have not previously been used for stable expression of α_2 adrenergic receptors, it was first necessary to determine the minimum concentration of antibiotic (G418) required for 98-99% cell death. By incubating JEG-3 cells with increasing concentrations of G418, it was determined that 0.5 mg/ml G418 in DMEM was adequate to kill a large majority of the control (non-tranfected) cell population.

Initial screens yielded no α_2-C2 clones and several clones expressing the α_2-C4 and α_2-C10 receptor subtypes. A second attempt to obtain a stably-transfected line expressing the α_2-C2 receptor subtype was made, and only a single line (C2-38) expressing ~0.5 pmole/mg was obtained. Other α_2-C2 cell lines expressing higher levels of receptor were not viable and could not be maintained. The α_2-C2 receptor was expressed at 0.4 pmoles/mg protein in JEG-3 cells and exhibited a high affinity for [^{3}H]rauwolscine (Kd=1.4 nM) (figure 28, table 3). Competition binding studies demonstrated that the α_2 receptor expressed in this cell line possessed the pharmacology characteristic of the α_2-C2 receptor subtype (figure 29). The expressed α_2-C2 receptor exhibited a characteristically lower affinity for yohimbine (K_i=8.6 nM) than either the α_2-C4 or α_2-C10 subtypes (table 4). Moreover, the expressed α_2-C2 receptor exhibited a low affinity for oxymetazoline (K_i=547 nM), and an intermediate affinity for prazosin compared to either the α_2-C4 or α_2-C10

receptor subtypes. These radioligand binding data suggest that this cell line indeed expresses the α_2-C2 adrenergic receptor subtype.

A single α_2-C4 cell line (C4-25) was isolated and shown to exhibit very high affinity for [^{3}H]rauwolscine (K_D=0.125 nM) (figure30). This clonal line grew extremely well and expressed ~1.0 pmole/mg of α_2 adrenergic receptor. These cells exhibited extremely high affinity for yohimbine (K_I=0.4 nM) and as expected, fairly high affinity for the α_1 receptor antagonist prazosin (K_I=60 nM), while oxymetazoline was only slightly less potent (K_I=120 nM). The extremely high affinity for both yohimbine and rauwolscine, as well as the high affinity for prazosin suggest that these cells indeed express the α_2-C4 receptor subtype.

Two cell lines expressing the α_2-C10 adrenergic receptor, termed C10-10 and C10-14 were isolated and characterized. Although saturation analysis suggested that the the C10-10 line expressed ~6 pmoles/mg of receptor (table 3), subsequent radioligand binding experiments revealed that the actual expression level was 2-3 pmoles/mg. The receptors expressed in these cells possessed somewhat lower affinity for [^{3}H]rauwolscine (K_D=3.3 nM) than either the α_2-C2 and α_2-C4 cell lines, however this value agrees closely with previous radioligand binding studies with this receptor subtype (Fraser *et al.*, 1989). The α_2 receptor expressed in these cells exhibited the pharmacology characteristic of the α_2-C10 receptor subtype (figure 33), including very high affinity for the agonist oxymetazoline (K_I=9.3 nM) and extremely low affinity for the antagonist prazosin (K_I=1866 nM). The C10-14 cell line exhibited a similar pharmacologic profile to the C10-10 line (table 4), with very low affinity for prazosin and high affinity for oxymetazoline (figure 35). These cells

expressed at a slightly lower level (B_{MAX}~1.5 pmole/mg) as compared to the C10-10 line. Based on these data, both the C10-10 and C10-14 cell lines appear to express the cloned α_2-C10 (α_{2A}) receptor subtype.

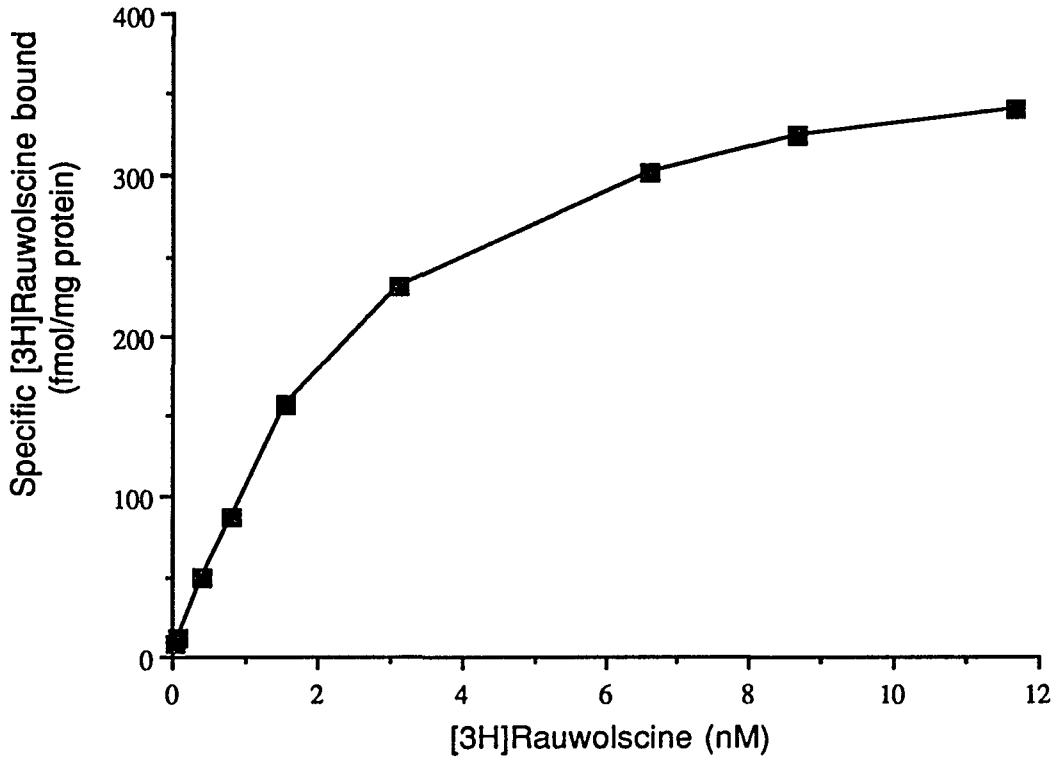


Figure 28. Saturation binding of [^{3}H]rauwolscine to membranes from stably-transfected α_2 C2-38 cells. Samples were incubated for 1 hour at 25°C, and reactions terminated by rapid filtration onto Whatman GF/B filters followed by three washes with ice cold sodium-potassium phosphate buffer. Non-specific binding was determined by interpolation of non-specific binding at 0.5, 2 and 8 nM concentrations.

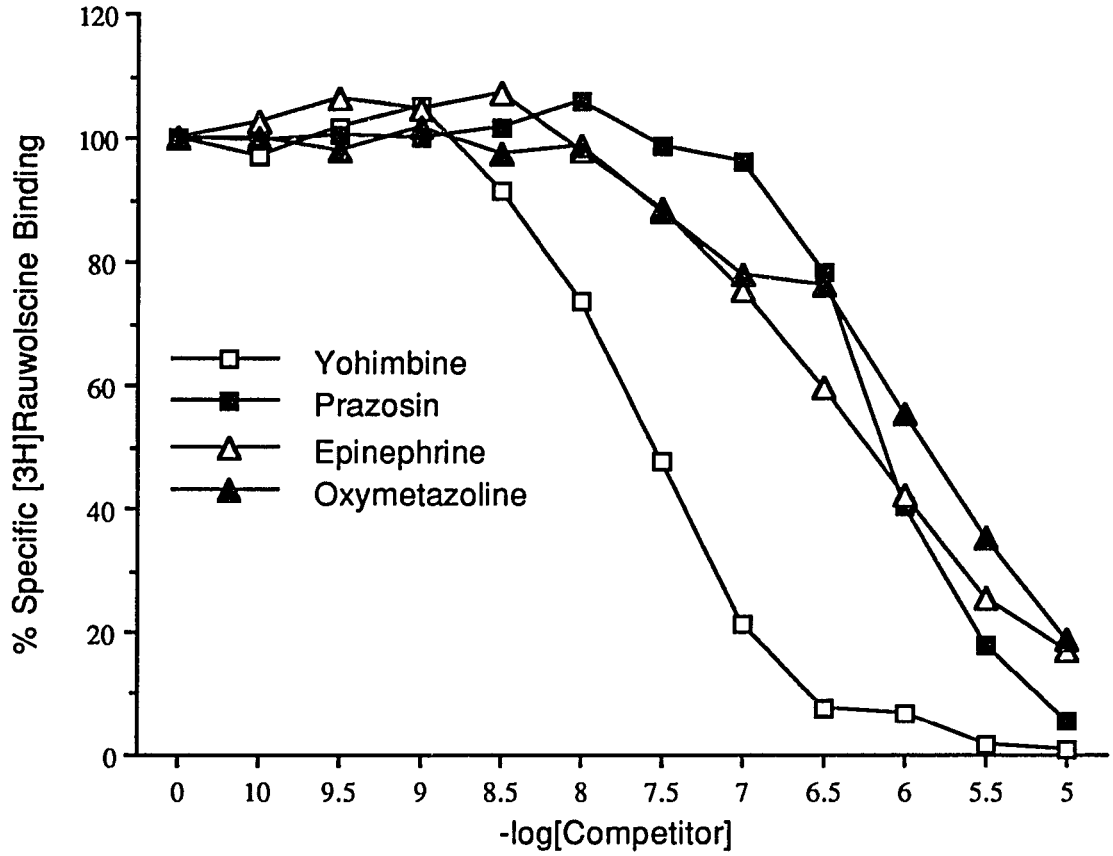


Figure 29. Competition by adrenergic ligands for binding of [^{3}H]rauwolscine to membranes from α_2 C2-38 cells. [^{3}H]Rauwolscine was used at 2 nM final concentration. Samples were incubated as described in figure 28. For competition studies, non-specific binding was determined in the presence of 10 µM yohimbine.

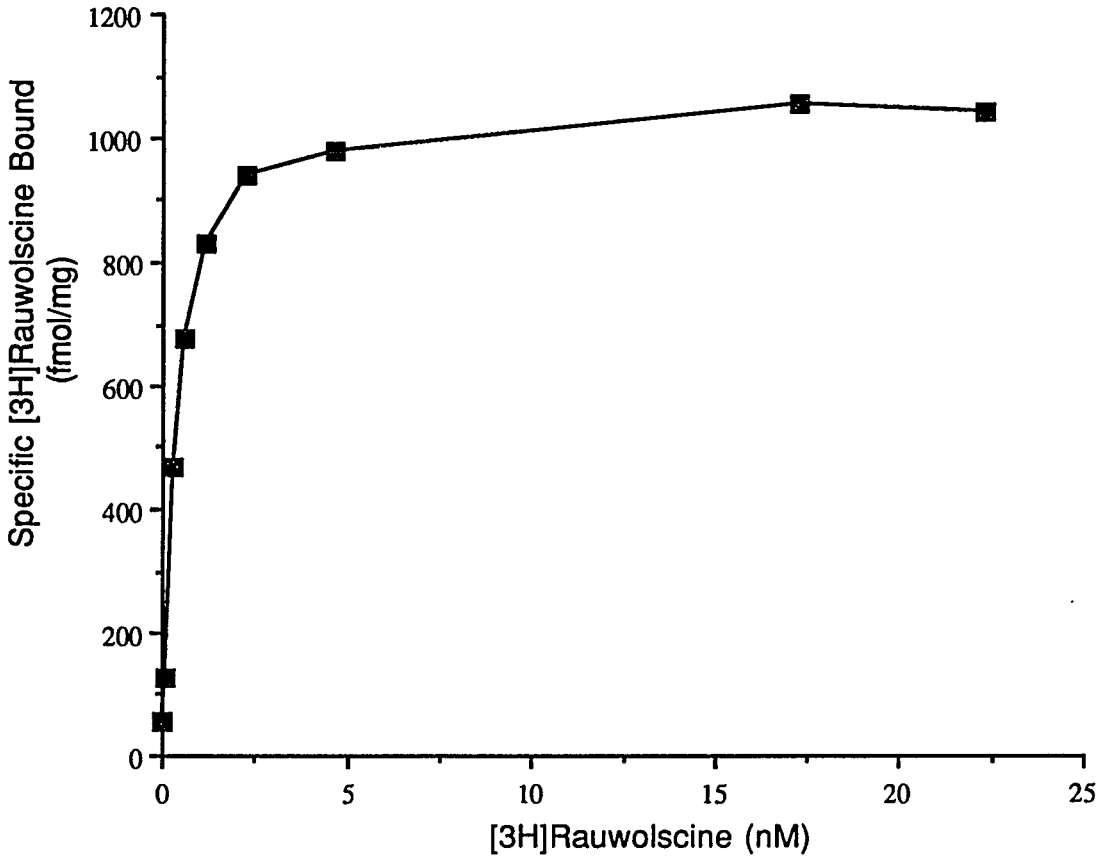


Figure 30. Saturation binding of [^{3}H] rauwolscine to membranes from stably-transfected α_2 C4-25 cell line. Non-specific binding was determined as described in figure 28.

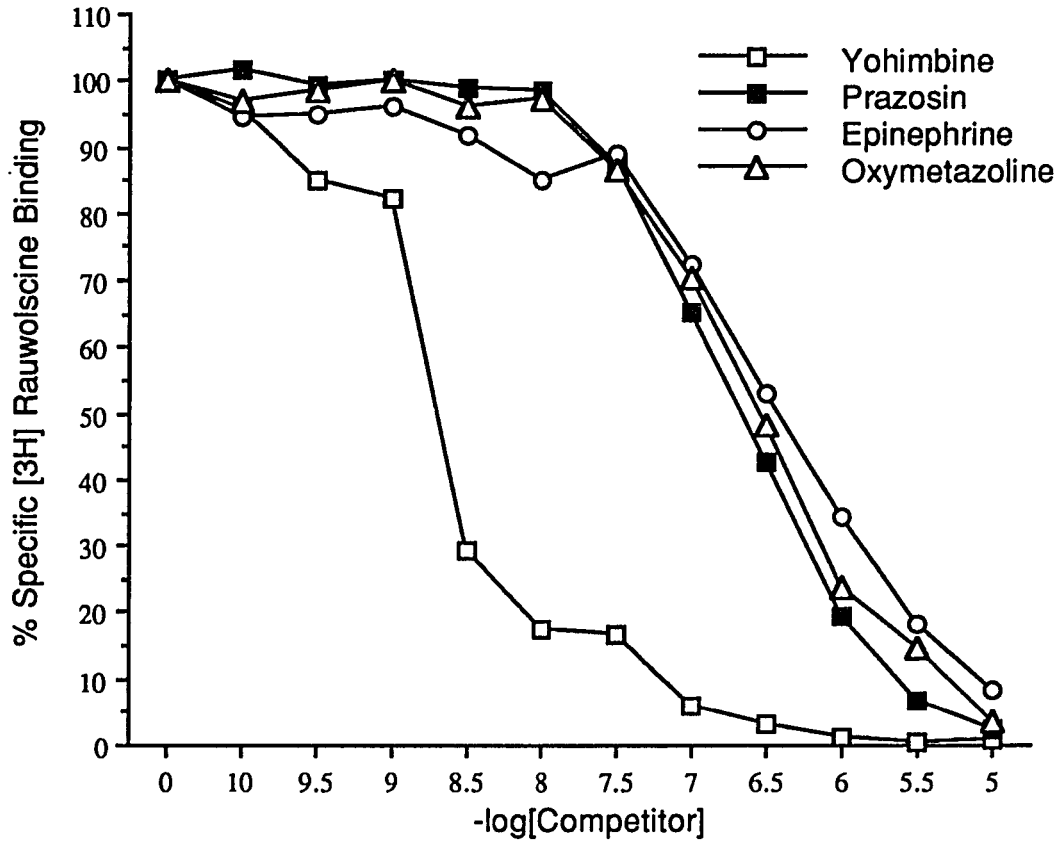


Figure 31. Competition by adrenergic ligands for binding of 1 nM [^{3}H] rauwolscine to membranes from α_2 C4-25 cell line. Non-specific binding was determined as described in figure 29.

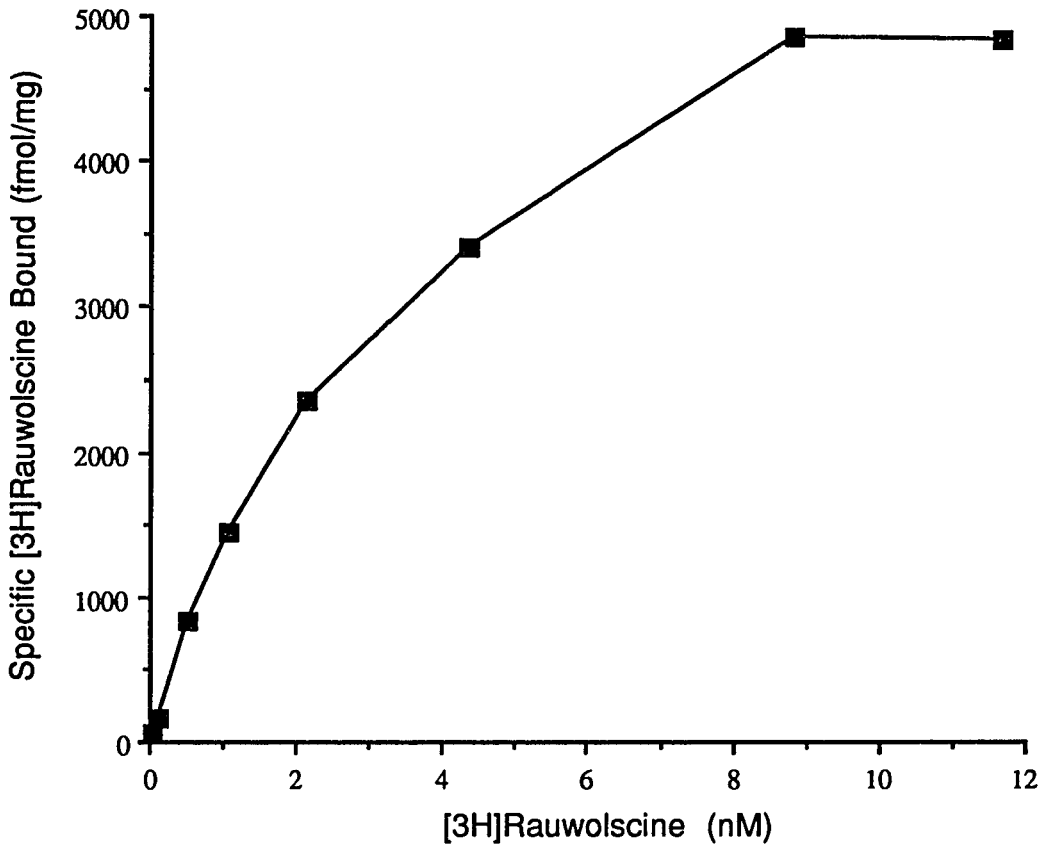


Figure 32. Saturation binding of [^{3}H]rauwolscine to membranes from stably-transfected α_2 C10-10 cell line. Non-specific binding was determined as described in figure 28.

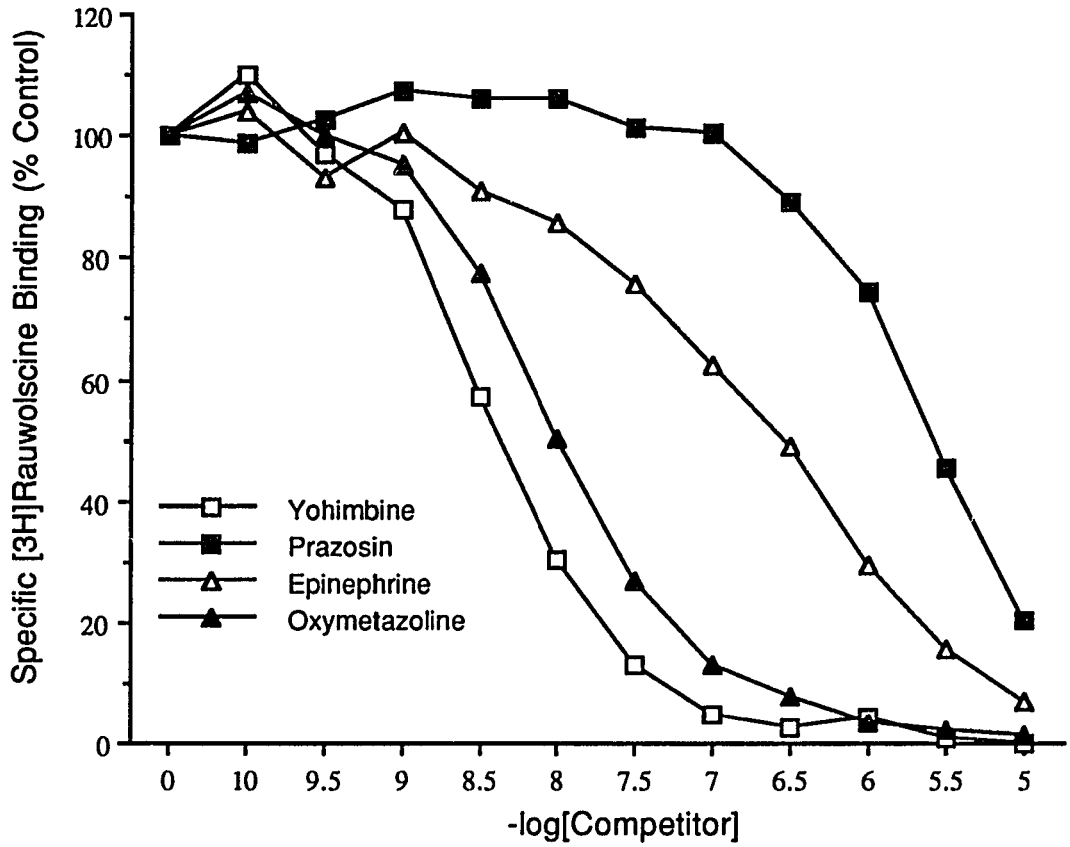


Figure 33. Competition by adrenergic ligands for binding of 0.5 nM [^{3}H]rauwolscine to membranes from α_2 C10-10 cells. Non-specific binding was determined as described in figure 29.

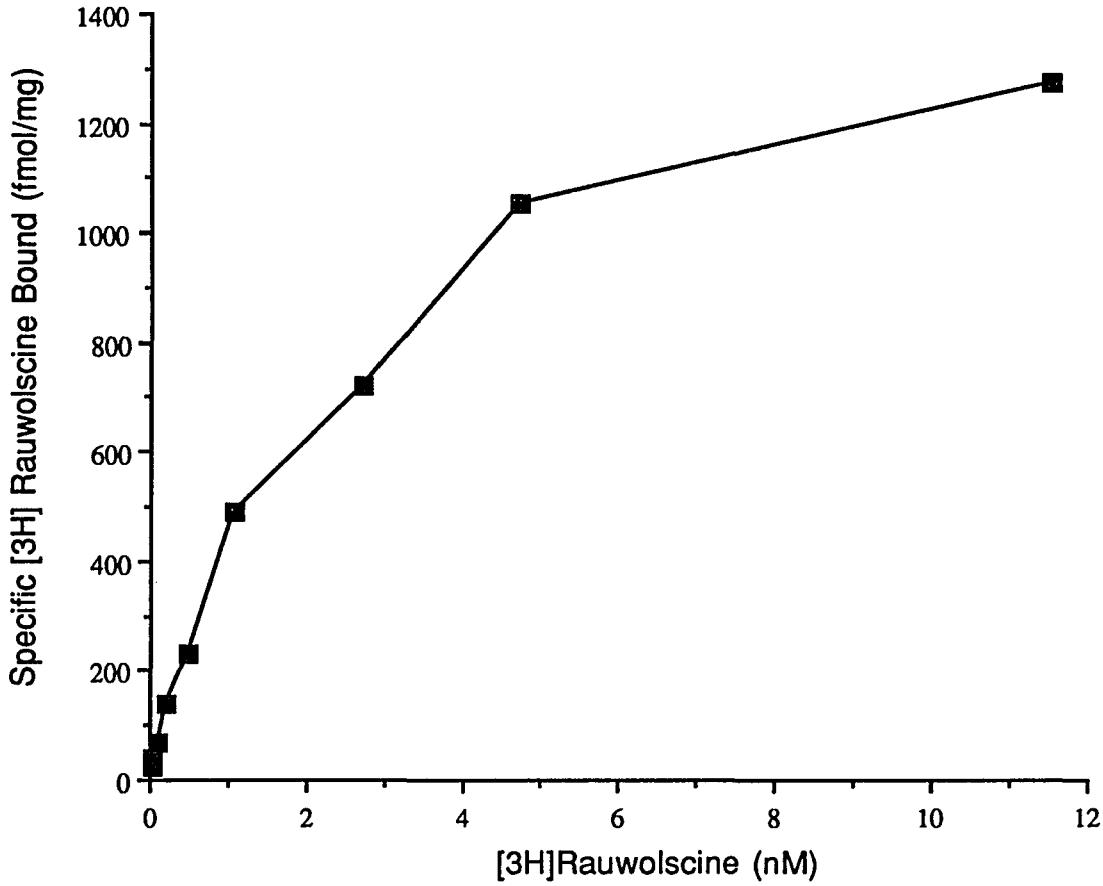


Figure 34. Saturation binding of [^{3}H]rauwolscine to membranes from stably-transfected α_2 C10-14 cells. Non-specific binding was determined as described in figure 28.

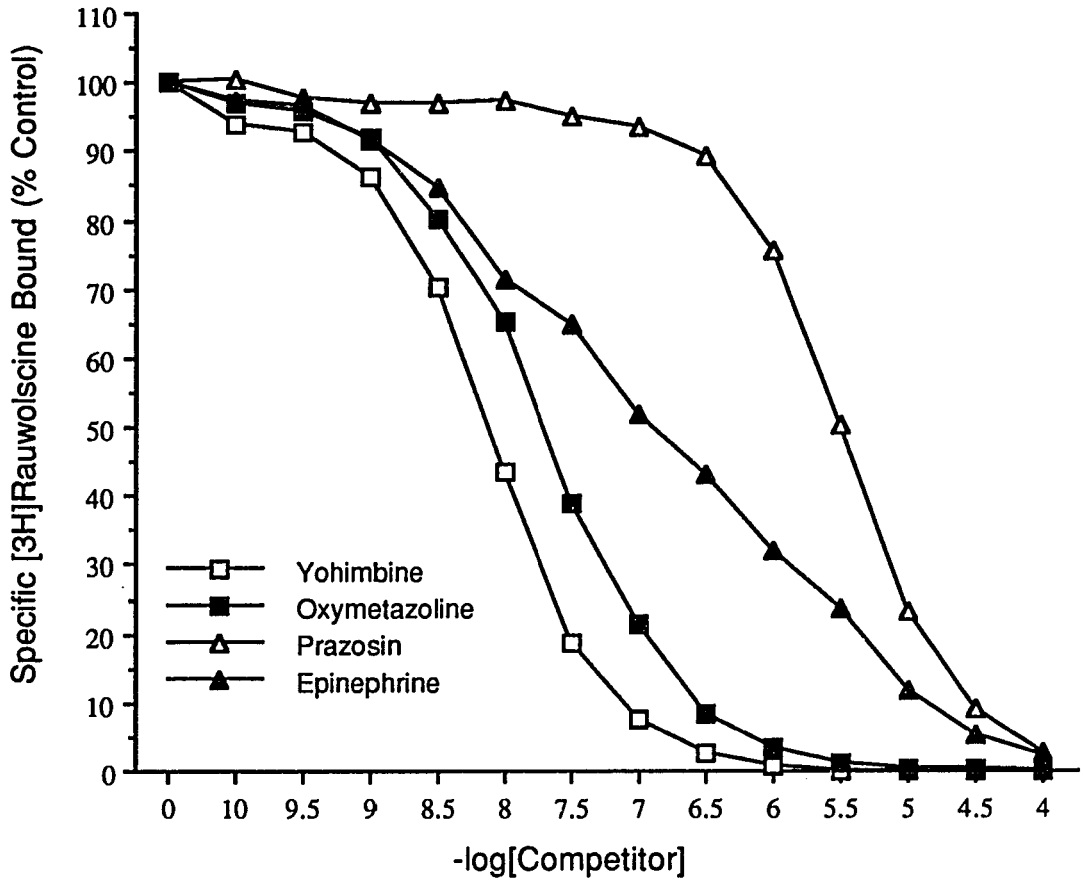


Figure 35. Competition by adrenergic ligands for binding of 0.5 nM [^{3}H]rauwolscine to membranes from stably-transfected α_2 C10-14 cells. Non-specific binding was determined as described in figure 29.

Table 3

B_{max} AND K_D VALUES FOR SATURATION BINDING OF [^{3}H] RAUWOLSCINE TO MEMBRANES FROM STABLY-TRANSFECTED JEG-3 CELLS EXPRESSING THE HUMAN α_2-ADRENERGIC RECEPTOR SUBTYPES

Cell Line	B_{max} (pmol/mg)	K_D (nM)
C2-38	0.42	1.40
C4-25	1.0	0.17
C10-10	6.0	3.28
C10-14	1.54	2.42

*B_{max} and K_D values were determined by saturation radioligand binding using 10-15 nM [^{3}H]rauwolscine. Non-specific binding was determined by interpolation of non-specific binding at 0.5, 2 and 8 nM rauwolscine. B_{max} and K_D values were calculated using the INPLOT4 program.

TABLE 4

K_I VALUES FOR COMPETITION BY ADRENERGIC LIGANDS FOR BINDING OF [^{3}H]RAUWOLSCINE TO MEMBRANES FROM STABLY-TRANSFECTED JEG-3 CELLS EXPRESSING THE HUMAN α_2 ADRENERGIC RECEPTOR SUBTYPES

	Ki (nM)*			
Compound	C2-38	C4-25	C10-10	C10-14
Yohimbine	8.6	0.4	3.0	5.1
Oxymetazoline	542	120	9.3	14.4
Epinephrine	137	60	224	162
Prazosin	272	60	1866	2592

*Ki values were determined from competition radioligand binding data shown according to Cheng and Prussof, (1973).

4.3.2 cAMP-dependent reporter gene studies

In order to quickly determine if the receptors in these cells coupled adenylyl cyclase, reporter plasmid pCRE(+) alone was transfected into these cells, and we attempted to measure a functional response using the CAT reporter gene system. In a limited number of experiments, addition of epinephrine to C2-38 cells produced a purely stimulatory response (figure 36), suggesting that even in these stably-transfected cells, at low levels of receptor expression, the α_2-C2 receptor still potentiates cAMP production. With time however, expression of the α_2-C2 receptor in these cells declined and subsequently, they no longer proved useful. While we were capable of isolating several α_2-C4 and α_2-C10 expressing cell lines, only a few α_2-C2 cell lines were initially isolated. In addition, only those cell lines expressing very low (<0.5 pmoles/mg) of receptor were viable for any length of time. It would appear then, that long-term expression of the α_2-C2 receptor subtype in JEG-3 cells may have deleterious effects on cell viability.

Studies with the α_2-C4 cell line (C4-25) were somewhat more successful. Addition of epinephrine to forskolin-treated C4-25 cells transfected with the reporter plasmid elicited a dose-dependent inhibition of reporter gene expression (figure 37). This result was replicated several times, and demonstrates that the α_2-C4 receptor expressed in these cells still functions in a purely inhibitory manner with respect to reporter gene expression.

Unfortunately, similar studies with the α_2-C10 cell lines using the reporter gene were not as successful. Reporter plasmid pCRE(+) was introduced into both C10-10 and C10-14 cell lines, and attempts were made to

measure the effect of epinephrine on forskolin-stimulated CAT activity. Unlike results with the C4-25 cells, we were unable to measure substantial amounts of either basal or forskolin-stimulated CAT activities in these α_2-C10 cell lines. Where parallel transfections of control, untransfected JEG-3 cells would yield ~1000 CPM of basal CAT activity with a 4-5 fold stimulation, similar transfections of these α_2-C10 cell lines would elicit only 100-200 cpm of CAT activity which did not respond significantly to forskolin.

Since it was unclear whether the α_2-C10 cell lines still possessed functional adenylyl cyclase, we proceeded to measure basal and forskolin-stimulated adenylyl cyclase activity in membranes from these cell lines. Cell membranes were prepared and were assayed for adenylyl cyclase activity by monitoring conversion of [^{32}P]ATP to cAMP (DeVivo and Maayani, 1985). Basal activities were very low in both control JEG-3 cells as well as in C10-10 and C10-14 cells. Addition of 10 μM forskolin elicited an 6-fold increase in adenylyl cyclase activity in control cells and a similar increase in stably-transfected C10-14 cells (data not shown). Adenylyl cyclase activity was also assessed in C10-10 cells. Again, basal activities were low in both control JEG-3 cells and in C10-10 cells. Forskolin (10 μM) produced an 8-fold stimulation in adenylyl cyclase activity and nearly an 11-fold increase in C10-10 cell membranes (data not shown). These results imply then, that adenylyl cyclase is present and responsive in these cell lines and that loss of the adenylyl cyclase cannot account for the distinct lack of CAT activity observed in these cells. Since adenylyl cyclase activity itself was unaffected, this distinct lack of CAT activity may indeed reflect some event downstream further still from cAMP.

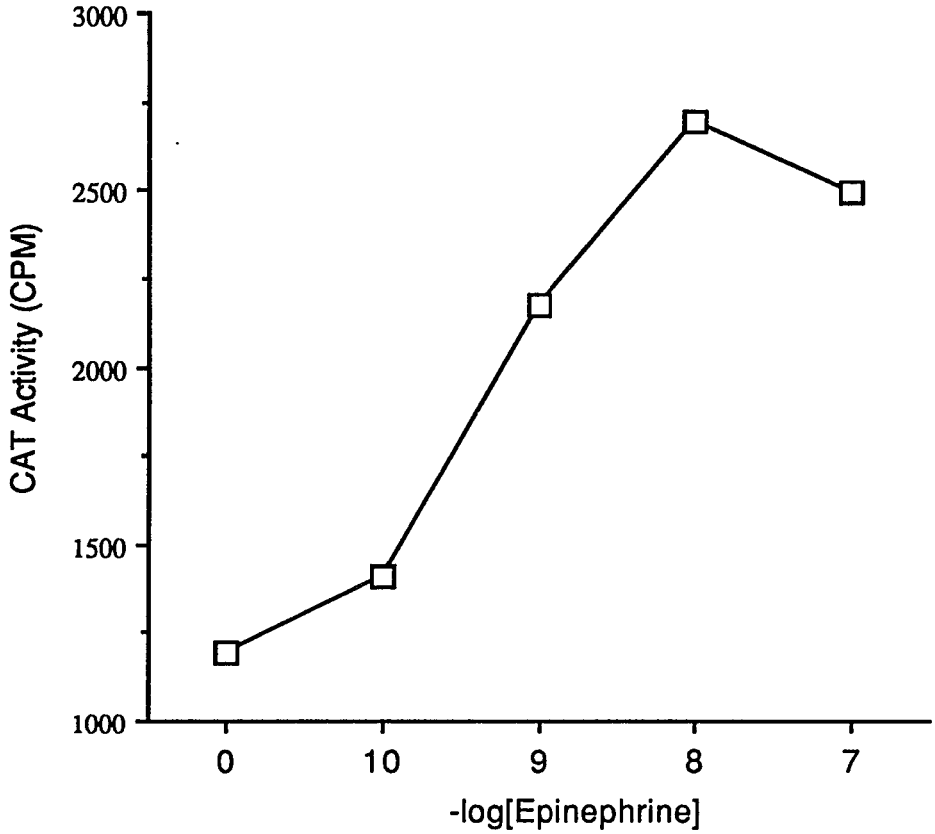


Figure 36. Effect of agonist epinephrine on cAMP-dependent CAT activity in stably-transfected C2-38 cells transfected with reporter plasmid pCRE(+). Each point represents a single plate of transfected cells.

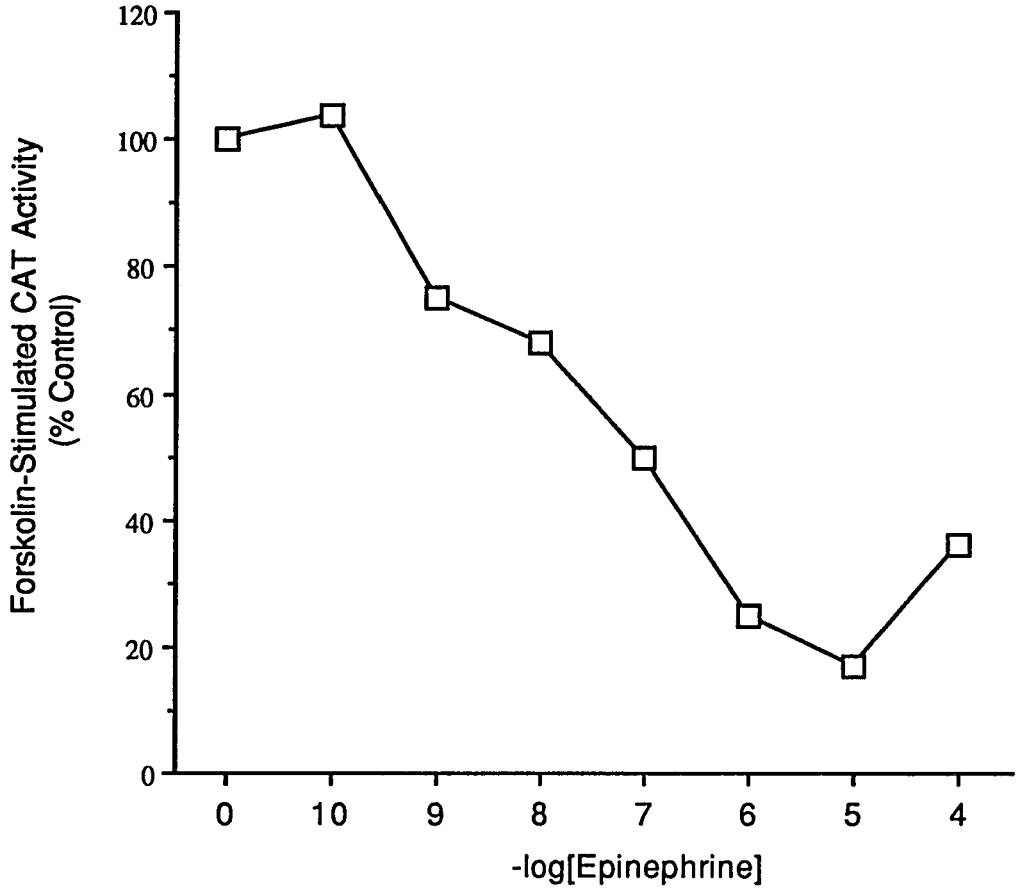


Figure 37. Effect of epinephrine on forskolin-stimulated CAT activity in stably-transfected C4-25 cells transfected with reporter plasmid pCRE(+). Each point represents the mean of two individually-transfected plates.

4.3.3 Direct cAMP Measurement Studies

Since we were unable to successfully measure CAT activity in several of these stably-transfected cell lines, we proceeded to use an intact-cell cAMP assay (Gilman, 1970) to quantify cAMP production. We first needed to establish optimal conditions for observing inhibition of forskolin-stimulated cAMP production in the JEG-3 cells. A forskolin dose-response curve was performed in untransfected JEG-3 cells to determine the optimal dose necessary for stimulating cAMP production. Short, three minute incubations with forskolin elicited a dose-dependent increase in cAMP production in JEG-3 cells, with maximal stimulation of 150-fold at 100 μM (figure 38). This result essentially mirrors the effect of forskolin on CAT activity JEG-3 cells transfected with the reporter plasmid (figure 8), thus providing evidence that the CAT system closely reflects intracellular cAMP levels on a transient basis.

We then performed agonist dose response curves in stably-transfected C4-25 cells as well as both C10-10 and C10-14 cells. Using 10 μM forskolin to stimulate cAMP production over a three minute time period, we did not consistently observe dose-dependent inhibition of cAMP production. Subsequently, we proceeded to use longer 15-minute incubations with only 1 μM forskolin. Figure 39 shows the effect of agonists medetomidine, p-aminoclonidine and epinephrine in JEG-3 cells stably-transfected with the α_2-C4 adrenergic receptor. Each agonist produced nearly complete inhibition of forskolin-stimulated cAMP production in these cells. Medetomidine was the most potent agonist, with an EC_{50} value of 1 nM (table 5). PAC exhibited intermediate affinity (EC_{50}=50 nM), while epinephrine was the least potent in

these cells (EC_{50}=400 nM). This result essentially confirms our results using the transient CAT assay system, that agonists produce purely inhibition of cAMP production in JEG-3 cells expressing the α_2-C4 adrenergic receptor subtype.

We then examined the effect of these agonists on forskolin-stimulated cAMP production in stably-transfected C10-14 cells. This cell line was chosen since the receptor expression level (~1.5 pmoles/mg) was most comparable to the C4-25 cell line (~1.0 pmole/mg). Again, each agonist elicited purely dose-dependent inhibition of forskolin-stimulated cAMP production (figure 40). The overall order of potency was identical to that observed in the C4-25 cells. Medetomidine again was the most potent (EC_{50}=0.8 nM), followed by PAC (EC_{50}=6.3 nM) and epinephrine, which displayed very low potency (EC_{50}=10 μM). In contrast to our observations with the transient CAT system, we did not observe any reversal of inhibition, or potentiation of forskolin-stimulated cAMP production in these stably-transfected cells. Rather, each agonist elicited a purely inhibitory effect on cAMP production. A similar pattern was observed for several of these agonists in the C10-10 cell line as well, such that even at higher levels of stable expression, the α_2-C10 receptor subtype appears to behave in a purely inhibitory manner with respect to cAMP.

To summarize our results with the stably-transfected JEG-3 cells, we have demonstrated that both the α_2-C4 and α_2-C10 receptor subtypes may be stably-expressed at relatively high levels in JEG-3 cells. Stable expression of the α_2-C2 adrenergic receptor subtype in these cells appears to have deleterious effects on cell survivial and viability. Each subtype however, exhibited the pharmacology characteristic of its own unique subtype. With

respect to cAMP production, incubation of cells with forskolin produced a dose-dependent increase in cAMP levels. Agonists elicited purely inhibition of forskolin-stimulated cAMP in both α_2-C4 and α_2-C10 cell lines. Thus, these studies suggest that the α_2-C2 receptor subtype still couples exclusively to increases in cAMP, whereas both α_2-C4 and α_2-C10 appear to couple primarily to inhibition of cAMP production.

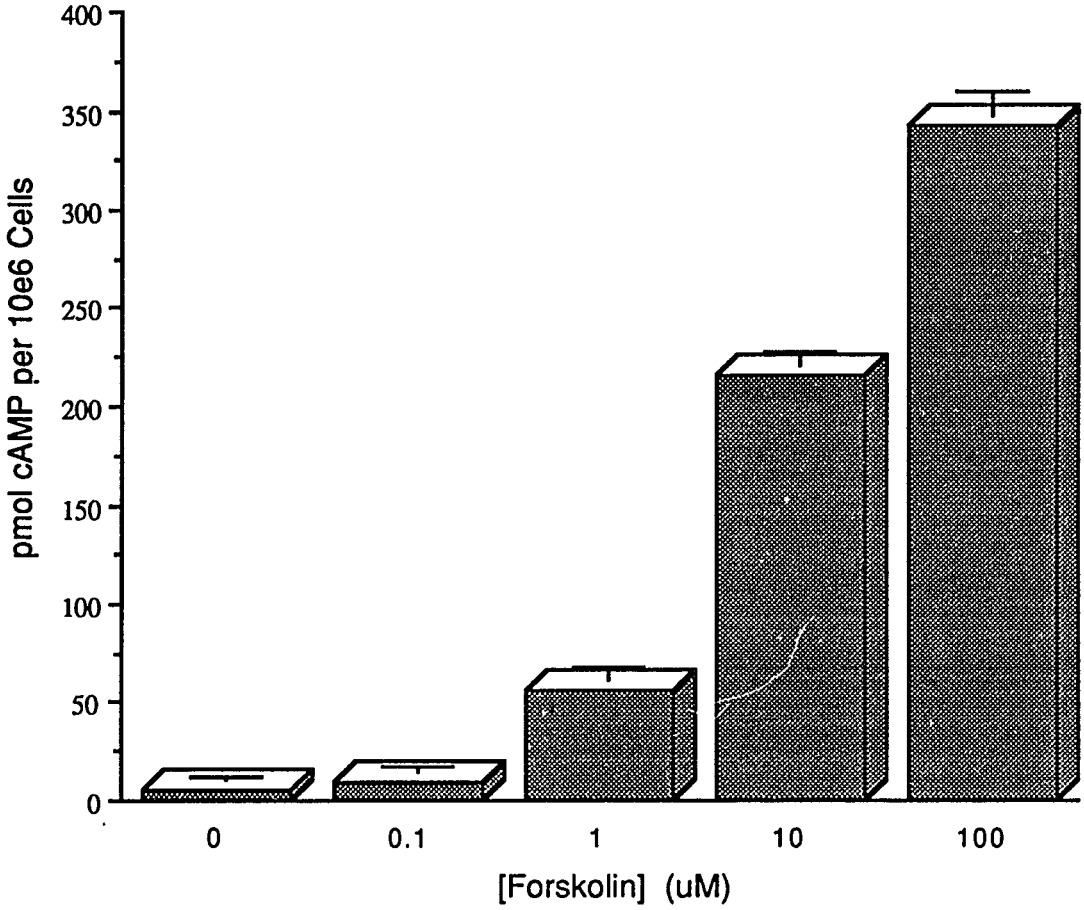


Figure 38. Forskolin stimulation of cAMP production in untransfected JEG-3 cells. Cells were pre-incubated with 0.1 mg/ml IBMX for 1 minute, then incubated with increasing concentrations of forskolin for three minutes at 37°C.

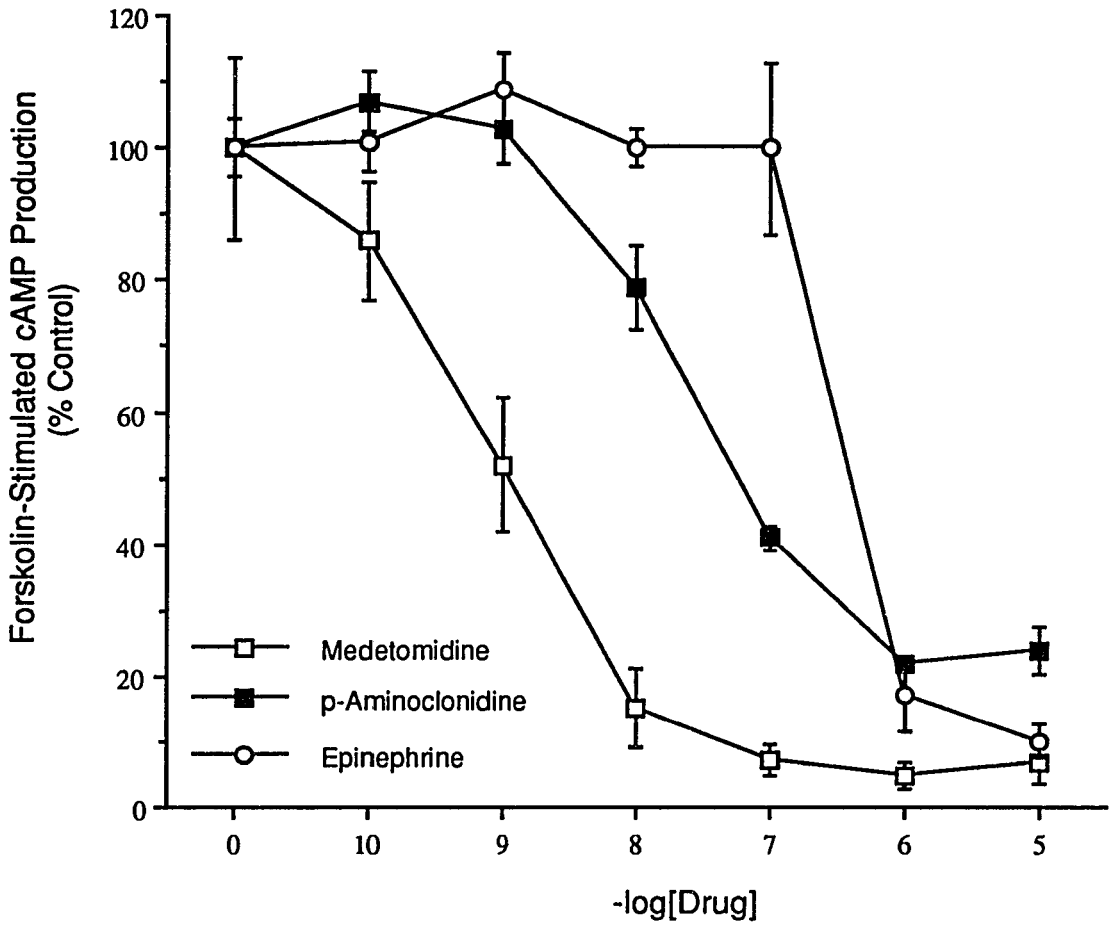


Figure 39. Effect of agonists medetomidine, p-aminoclonidine and epinephrine on forskolin-stimulated cAMP production in stably-transfected α_2 C4-25 cells. Cells were pre-incubated with IBMX for two minutes, then exposed to 1 μM forskolin and drugs for 15 minutes at 37 °C.

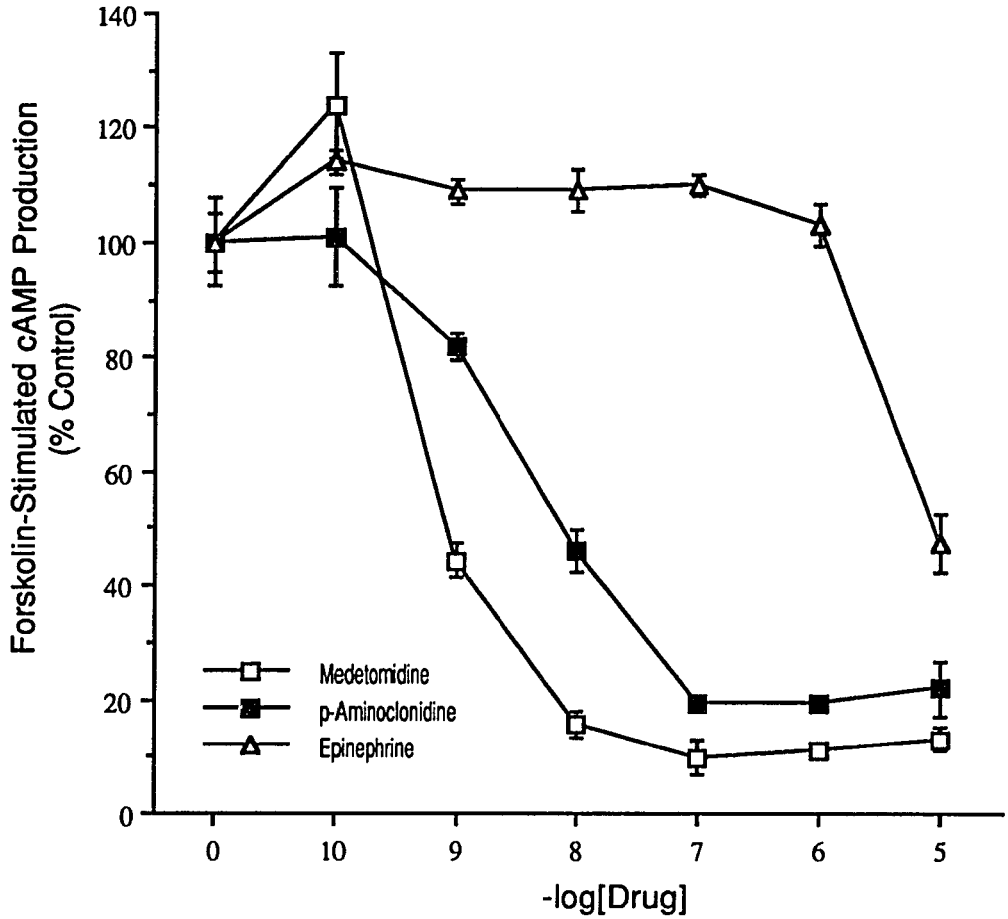


Figure 40. Effect of agonists medetomidine, epinephrine and p-aminoclonidine on forskolin-stimulated cAMP production in stably-transfected α_2 C10-14 cells. Cells were incubated as described in figure 39.

Table 5

AGONIST POTENCIES FOR INHIBITION OF FORSKOLIN-STIMULATED cAMP PRODUCTION IN STABLY-TRANSFECTED JEG-3 CELLS EXPRESSING THE α_2-C4 AND α_2-C10 ADRENERGIC RECEPTOR SUBTYPES

Agonist	EC_{50} (nM)	
	α_2-C4	α_2-C10
Medetomidine	1.0	0.8
p-Aminoclonidine	50.0	6.3
Epinephrine	400	10,000

4.4 Discussion

In these studies we have sought to develop JEG-3 cell lines which stably-express the human α_2 adrenergic receptor subtypes. Previous studies have demonstrated that these receptors can be stably-expressed in numerous cell types, including CHO (Fraser *et al.*, 1989), S115 mouse mammary tumor cells (Jansson *et al.*, 1994) and NIH3T3 fibroblasts (Duzic *et al.*, 1992). Since our results suggest that these receptors are functionally distinct in the JEG-3 cells, we felt it necessary to attempt stable expression of these receptors in the same cell line. In this way, we might better mimic the conditions in the endogenously-expressing tissues. The JEG-3 cells are a human cell line derived from a placental tumor, and express high levels of human chorionic gonadotropin (Kohler and Bridson, 1971). In our earlier studies, we have shown that these cells can be transiently transfected with α_2 adrenergic receptor-coding plasmids and will express low levels (0.5-1 pmole/mg) of receptor protein.

In the present studies, we have shown that the α_2 adrenergic receptors can also be stably-expressed in the JEG-3 cell line. While high-level, long-term expression of the α_2-C4 and α_2-C10 subtypes was obtained, we were unable to successfully express the α_2-C2 receptor subtype at levels above 0.5 pmoles/mg protein. Clonal lines expressing higher levels of α_2-C2 receptor were initially obtained, but were not viable, particularly those expressing extremely high levels of receptor (>3 pmoles/mg). These results, and the inability to maintain expression in the C2-38 cells suggest that expression of this receptor subtype is deleterious to cell survival.

We did however demonstrate that agonists still produce a purely stimulatory response in C2-38 cells, at least with respect to CAT activity. Whether agonists are capable of inhibiting forskolin-stimulated cAMP production still remains unclear. However, our inability to observe any inhibitory activity following agonist activation of this receptor suggests that the α_2-C2 will not inhibit adenylyl cyclase in the JEG-3 cells. If the α_2-C2 receptor is indeed purely stimulatory with respect to cAMP production in the JEG-3 cells, then perhaps chronically elevated cAMP levels may be involved in our inability to stably express this subtype. Further attempts at developing stably-transfected cells expressing the α_2-C2 will certainly be necessary to address this question.

All three receptor subtypes expressed in the JEG-3 cells exhibited characteristically high affinity for [^{3}H]rauwolscine, with the α_2-C4 having the highest affinity (table 3). Neither the α_2-C4, nor α_2-C10 appeared to have deleterious effects on JEG-3 cell viability, and expression was maintained for the duration of our experiments. Each receptor subtype, including α_2-C2, exhibited a pharmacological profile characteristic of its unique subtype. As expected, the α_2-C2 exhibited extremely low affinity for oxymetazoline, while the α_2-C10 bound this compound with relatively high affinity, characteristic of an α_{2A} receptor. The α_2-C4 receptor expressed in these cells exhibited much higher affinity for prazosin than either α_2-C2 or α_2-C10 receptor subtypes. In fact, K_i values for binding agreed closely with previous radioligand binding studies for all three subtypes (Bylund *et al.*, 1992).

With respect to cAMP production, we have demonstrated that the JEG-3 cells respond rapidly and robustly to forskolin (figure 38). Forskolin produced

a substantial increase in cAMP production even following a 3-minute incubation (figure 38). In fact, forskolin produced virtually identical effects on both cAMP production and reporter gene expression (figure 8). By comparing these two dose-response curves, it is clear that CAT activity provides a good indicator of intracellular cAMP. While cAMP production is a rapid, short term response, the CAT activity is measured following a 4-hour incubation with drug. Since CAT activity appears to so closely mimic cAMP levels, we may actually be measuring a steady-state level of cAMP, in equilibrium between production by adenylyl cyclase and degradation by phosphodiesterase. In any case, these data imply that CAT activity indeed provides a convenient, indirect measure of relative intracellular cAMP levels.

In terms of cAMP production, our results for the α_2-C4 essentially confirm our data from the transient system. Each agonist produced dose-dependent inhibition of forskolin-stimulated cAMP production. When compared to inhibition of forskolin-stimulated CAT activity, agonist potencies for inhibition of cAMP production in the stably-transfected cells were substantially lower. For example, the EC_{50} of epinephrine for inhibition of CAT activity in α_2-C4 transfected cells was 20 nM, whereas in stably-transfected cells the EC_{50} was 400 nM. For the α_2-C10, this difference in potencies between the two systems was even more pronounced. Thus, for inhibition of forskolin-stimulated CAT activity, the EC_{50} for epinephrine was 0.2 nM, while for inhibition of forskolin-stimulated cAMP production the EC_{50} was 10,000 nM. With respect to agonist potencies in the two systems then, the transient CAT system appears much more sensitive to agonist than the stable expression system. This enhanced sensitivity to agonist observed in

the transient system may reflect the relatively high level of expression within the transfected cell population.

When compared with other recent studies in which cAMP levels were similarly measured, agonist potencies in our system were significantly higher (Jansson *et al.*, 1994). For example, the EC_{50} of medetomidine for decreasing cAMP at the α_2-C10 in stably-transfected mouse S115 cells is 3.6 μM, whereas the EC_{50} for medetomidine at the α_2-C10 in stably-transfected JEG-3 cells was only 0.8 nM. Similarly for the α_2-C4, the EC_{50} of medetomidine for inhibition of cAMP production in S115 cells was 420 nM (Jansson *et al.*, 1994), compared to only 1 nM in JEG-3 cells.

In contrast to our results with the transient CAT system, we did not observe any reversal of inhibition or potentiation of forskolin-stimulated cAMP production in stably-transfected α_2-C10 cells. Receptor expression levels in our system were comparable to those in CHO cells in which agonist-induced potentiation of cAMP production was observed (Eason *et al.*, 1992). For the α_2-C10 receptor subtype then, the ability to couple to the cAMP stimulatory pathway appears to depend on its level of expression, whereas for the α_2-C2, coupling to stimulation of cAMP production in JEG-3 cells may occur even at low levels of receptor. Since agonist-induced potentiation of CAT activity was never observed in α_2-C4 transfected cells, we can conclude that this receptor subtype does not couple significantly to stimulation of cAMP production in JEG-3 cells. It would appear then, that the CAT assay system indeed closely mimics intracellular cAMP levels, and that the differences observed between the human α_2 receptor subtypes are inherent to their unique structure and affinity for G-proteins.

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

5.1 Future Directions

The present body of work clearly demonstrates our ability to functionally express α_2 adrenergic receptors, and examine pharmacology and effector coupling in a transient fashion. By developing a transient expression system, we have dramatically decreased the turnaround time required for studying cloned receptor function. More importantly however, this work attempts to provide a framework for future functional studies of G-protein coupled receptors. First, using this transient expression system, we can now quickly examine the effect of mutation on α_2 adrenergic receptor function. With respect to specific mutagenesis studies involving α_2 adrenergic receptors, previous results with the β_2 adrenergic receptor have demonstrated that palmitoylation (fatty acid acylation) of a carboxyl-terminal cysteine residue is required for coupling to adenylyl cyclase (O'dowd *et al.*, 1989). Palmitoylation of this residue is thought to form an additional intracellular loop which may be involved in coupling to G-proteins. Whereas all other α_2 receptors similarly possess a cysteine residue at this site, the α_2-C4 receptor exhibits a phenylalanine. Subsequently, mutant α_2-C4 receptors containing phe->cys as well as phe->gly mutations have been prepared (Porter and Regan, manuscript in preparation), and will be examined using this transient functional system. With respect to other potential mutations, studies with prostanoid EP_3 receptors have demonstrated the importance of the carboxyl

terminal segment in determining G-protein coupling specificity (Namba *et al.*, 1993). In the present studies, we have shown that the α_2-C2 and α_2-C4 receptor subtypes are inversely coupled to adenylyl cyclase. Thus, studies are currently underway to prepare chimeric α_2-C2/α_2-C4 adrenergic receptors in which the carboxyl tails have been switched between these two subtypes. Using the transient CAT reporter gene system, we might then determine if the carboxyl tails of these receptors are involved in directing specific G-protein interactions. Hopefully, these studies will help define specific regions of the receptor(s) involved in coupling to G_i vs. the stimulatory G-protein, G_s. Using transient expression, we should also be capable of co-expressing selected receptors and G-proteins in a single system in order to define precise molecular interactions (Conklin *et al.*, 1992).

This transient gene expression will also prove useful for functional studies of other G-protein coupled receptors as well. Already, this reporter gene system has been used to examine the functional coupling of prostanoid EP_3 receptors to the adenylyl cyclase signal transduction pathway (Regan *et al.*, 1994). These receptors were transiently-expressed in the JEG-3 cell system and shown to inhibit forskolin-stimulated CAT expression similar the the cloned α_2-C4 adrenergic receptor. When one considers the sequence diversity between α_2 adrenergic and prostanoid receptors, it would appear that this reporter gene system will prove useful for virtually any receptor coupled to adenylyl cyclase.

Finally, future studies should address development of even easier and more sensitive transient methods for measuring receptor activity. Potentially, one could replace the CAT gene, the workhorse of reporter plasmids, with a

more sensitive gene such as luciferase. Firefly luciferase, a 550 amino acid monomeric protein catalyzes the oxidation of D-luciferin, leading to production of light (Gould and Subramani, 1988). This assay system is reportedly 100-1000 times more sensitive than the traditional CAT assays. Subsequently, one could use fewer cells and increase the number of data points per experiment. An even better alternative would involve linking the CRE to expression of a secreted protein, such as human growth hormone (hGH). The expressed hGH can be secreted by hormonally-responsive cells (such as JEG-3) directly into the culture media, where it can be continuously monitored by radioimmunoassay (Selden *et al.*, 1986). Since the protein is secreted directly into the media, scraping and lysing the cells would be unnecessary. Moreover, the ease and sensitivity of the hGH immunoassay would allow for rapid screening of numerous samples. Taken together, these steps should dramatically increase our ability to study α_2 adrenergic receptor function and pharmacology. Hopefully, this study offers some framework for future studies bent on understanding the structural basis of α_2 adrenergic receptor function.

5.2 Summary and Implications

In conclusion, we have demonstrated that α_2 adrenergic receptors can be both transiently and stably expressed in human choriocarcinoma (JEG-3) cells, and that activation of these transiently-expressed receptors can regulate cAMP-dependent reporter gene activity. This reporter gene expression is highly cell-type specific, and appears to closely mimic intracellular cAMP levels. We have also shown that despite close structural and pharmacological similarity, expression of the three human α_2 adrenergic receptors can elicit unique intracellular responses. Furthermore, these responses appear to result from activation of distinct G-proteins within the transfected cell population.

The JEG-3 cells also provide a suitable host cell system for stable expression of α_2 adrenergic receptor subtypes. In these cells, both the α_2-C4 and α_2-C10 receptor subtypes solely inhibit forskolin-stimulated cAMP production, while the α_2-C2 receptors appear only to stimulate cAMP production . Unlike the stable expression system, this transient system reveals distinct differences in the effector-coupling pattern of the human α_2 adrenergic receptor subtypes not previously appreciated in other cellular systems (Eason *et al.*, 1992). Whether these differences are dependent solely on the α_2 receptor subtypes or on some facet of the expression system is still presently unclear. However, this transient approach has clearly provided a more rapid approach for the functional analysis of cloned α_2 adrenergic receptor subtypes. In characterizing the functional responses to activation of the wild-

type α_2 adrenergic receptors, we have thus provided a framework for future structure-function analyses involving the α_2 adrenergic receptor subtypes.

Using this transient approach, we have also demonstrated the potential for α_2 adrenergic receptors, and possibly other G-protein coupled receptors to regulate gene expression. At present, hundreds of G-protein coupled receptors have been cloned, and many of the signal transduction systems to which they couple have been characterized (Probst *et al.*, 1992). The long-term cellular consequences of receptor activation however, have yet to be elucidated. These studies and those of other groups (Jones *et al.*, 1991) (Macnulty *et al.*, 1992) have demonstrated the potential for bi-directional signaling of α_2 adrenergic receptors. Molecular dissection of the various signal transduction pathways to which these receptors couple may soon reveal which of these pathways is physiologically relevant. More importantly, multifunctional signaling by G-protein coupled receptors should permit development of therapeutic agents which selectively couple receptors to specific 2nd messenger pathways within the cell, thus providing another level of specificity beyond the drug-receptor interaction.

APPENDIX A

CALCIUM PHOSPHATE AND DEAE-DEXTRAN MEDIATED TRANSFECTION PROTOCOLS

I. Calcium Phosphate Transfection Protocol

Reagents:

2x HEPES-Buffered Saline:
- 5g HEPES
- 8g NaCl
- 0.37g KCl
- 0.125g Na_2HPO_4: $2H_2O$
- 1g Glucose
- 425 ml H_20
- pH to exactly 7.05 with NaOH
- H_2O to 500 ml, sterile filter, freeze at -20°C.

250 mM $CaCl_2$:
- 3.678 g $CaCl_2$:$2H_2O$
- 100 ml H_2O
- Filter sterilize and freeze at -20°C.

DMEM/PS/5%FBS pH 7.1

DMEM/PS/5%FBS pH 7.4

DMSO (Tissue culture grade)

Phosphate Buffered Saline pH 7.4

Procedure:

1. Change media on 10 cm plates of cells. Add 9 ml of DMEM pH 7.1 +5% Fetal bovine serum (FBS).

2. Incubate for 2-3 hrs. at 37°C.

3. Prepare calcium phosphate precipitates. For each plate to be transfected use a single sterile 15 ml conical centrifuge tube. To each tube add 20 µg DNA and 500 µl 2x HBS buffer. Vortex to mix. While vortexing, slowly add 500 µl of 250 mM $CaCl_2$. Add 2-3 drops per second while vortexing at maximum speed.

4. Incubate precipitates at room temperature for 20 minutes.

5. With gente swirling, dropwise add 1 ml precipitate to a single plate of cells. Place in incubator for at least 4-5 hrs. Particles of calcium phosphate should be visible under the microscope at medium power. During this time, the culture media should turn slightly cloudly and somewhat orange.

6. Aspirate media and to each plate add 3 ml DMEM (pH7.1)/5%FBS+ 10% DMSO. Leave at room temperature for 3 minutes. Aspirate off DMSO solution and add 10 ml of DMEM/PS/5% FBS pH 7.4.

7. Place in incubator and harvest cells 2-3 days later.

II. DEAE-Dextran Mediated Transfection Protocol

Reagents:
10 mg/ml DEAE-Dextran (Pharmacia) in 1x phosphate buffered saline.

100 mg/ml chloroquine (Sigma). Sterile filter, aliquot into 1-ml portions, wrap in foil and freeze at -20°C.

Dulbecco's modified eagle medium (DMEM) (Gibco), containing 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco), and 5% fetalbovine serum (FBS) (Hyclone).

Phosphate buffered saline:
2.7 mM KCl
1.5 mM KH_2PO_4
0.5 mM $MgCl_2$
137 mM NaCl
8.1 mM Na_2HPO_4, pH 7.4

Protocol:
1. Grow COS-7 cells in 15 cm plates to sub-confluence for 2-3 days to insure cell stability.

2. Prepare DEAE-Dextran/DNA solution. For each plate mix 50 µg of plasmid DNA, 9.5 ml PBS and 500 µl stock DEAE-dextran solution. Place at 37°C.

3. Rinse each plate with 20 ml PBS and add 10 ml of DEAE-dextran/DNA solution. Incubate plates for 30 minutes at 37°C

4. Prepare chloroquine solution. For each plate, dilute 10 µl of stock 100 mM chloroquine into 10 ml of DMEM/5% FCS.

5. Aspirate off DEAE-dextran solution and add 10 ml of chloroquine solution to each plate. Incubate at 37°C for 2.5 hours.

6. Aspirate off chloroquine solution and add 10 ml of DMEM + 10% DMSO, and incubate for three minutes at room temperature.

7. Aspirate off DMSO solution and feed cells 30 ml of DMEM + 5% FBS and harvest cells 2-3 days later.

APPENDIX B

CELL LYSIS AND [^{3}H]CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ASSAY

I. Cell Lysis

Reagents:

TEN: 40 mM Tris pH 7.5
1 mM EDTA
150 mM NaCl

Phosphate Buffered Saline (PBS)

250 mM Tris pH 7.5 (cold)

Procedure:

1. Following transfection and drug incubation, aspirate medium from cells and rinse each plate twice in 5 ml PBS.
2. Aspirate off PBS and add 1 ml cold TEN, place plates at 4°C in refrigerator.
3. Scrape cells into TEN and pipette into a microfuge tube. Place tubes on ice. Spin down cells briefly in microfuge ~1 min.
4. Aspirate off TEN and resuspend cells in 200 µl cold 250 mM tris solution.
5. Freeze cell suspension in dry ice/ethanol bath five minutes, then thaw rapidly at 37°C. Repeat the freeze-thaw cycle two more times.
6. After final thaw, vortex tubes for 1-2 seconds and spin down membranes in microfuge for 2 minutes.
7. Transfer supernatant to a fresh tube. [^{3}H]CAT assays may be performed immediately or supernatants may be frozen at -20°C for several days without losing activity.

II. [^{3}H]Chloramphenicol Acetyl Transferase (CAT) Assay

Reagents:

[^{3}H]Chloramphenicol (CAM) (New England Nuclear #NET-928)

5 mg/ml Butyryl CoEnzyme A (Sigma #B-1508)

2 molar Tris-HCl pH 8.0 (room temperature)

10 mM Tris/1 mM EDTA buffer, pH 8.0 (TE)

Mixed xylenes (Aldrich)

Procedure:

1. Pre-extract chloramphenicol. Mix 20 µl [^{3}H]CAM (1 µCi/µl), 3.2 µl cold 100 mM CAM and 77 µl ethanol. Dilute to 1 ml with water. Divide into four-250 µl samples and to each add an additional 250 µl of water. To each 500 µl sample add 500 µl of mixed xylenes and vortex. Centrifuge samples briefly to separate phases. Remove the top xylene phase to liquid waste and repeat xylene extraction. Count 20 µl of final aqueous phase (should be about 150-200,000 CPM or 0.2 µCi).

2. Prepare Chloramphenicol assay mixture 50 µl/sample:

   20 µl [^{3}H] CAM (0.2 µCi)
   20 µl H_2O
   5 µl 5 mg/ml butyryl coenzyme A
   5 µl 2 molar Tris HCl pH 8.0

3. Aliquot 50 µl mixture into 1.5 ml microcentrifuge tube, add 50 µl cell cytosol. Mix gently by flicking tube, and incubate at 37°C for 1 hour.

4. Stop reaction by adding 200 µl mixed xylenes, vortex, centrifuge, and remove 180 µl xylenes to fresh tube. Back extract xylenes with 100 µl TE buffer, centrifuge, recover 160 µl xylenes, transfer to another tube extract and again similarly, recovering 140 µl xylene phase directly to a vial for scintillation counting.

APPENDIX C

SELECTION OF STABLY-EXPRESSING JEG-3 CELL LINES

Materials:
pRX-NEO (neomycin resistance plasmid) in sterile water

α_2 Adrenergic receptor coding plasmid

2x Hepes buffered saline (Appendix A)

250 mM calcium phosphate (Appendix A)

Dulbecco's Modified Eagle Medium (DMEM) pH 7.4 + penicillin/streptomycin

Dulbecco's Modified Eagle Medium (DMEM) pH 7.1 + penicillin/streptomycin

Dimethylsulfoxide (DMSO)

Vacuum grease (autoclaved)

Sterile forceps

Geneticin (G418) powder (Gibco #11811-031)

0.025% Trypsin solution in phosphate buffered saline

Procedure:

1. 1-2 Days prior to transfection, split a confluent dish of JEG-3 cells into three 12-well plates. To each well add 2 ml media and 0.5 ml of resuspended cells. Grow in DMEM/10% FCS.

2. When cells are ~50% confluent, change media to 2 ml DMEM pH 7.1, and incubate cells for about 2 hours.

3. During this time prepare transfection mixtures. For the 36 wells prepare eight 1 ml $CaPO_4$ precipitates. To each tube add 2 µg pRX-NEO DNA and 20 µg of receptor coding plasmid. Add 500 µl of 2x HBS.

4. Dropwise add 500 µl of 250 mM $CaCl_2$ while vortexing and incubate precipitates 20 minutes at room temperature. At this time add 200 µl of mixture to each well and incubate cells for five hours at 37°C

5. Prepare a solution of DMEM/pH 7.1/10% DMSO. Incubate cells with DMEM/DMSO solution for 3 minutes at room temperature, aspirate off and add 2 ml. DMEM/5% FBS and incubate for two days.

6. Prepare DMEM + geneticin antibiotic. Weigh out 0.5 g G418 and dissolve in 50 ml of DMEM. Sterile filter G418 solution through 0.2 μ filter and add 25 ml to each of two 500 ml bottles of media. Two days post-transfection change the media on all wells to DMEM/pH7.4/5% FCS + 0.5 mg/ml G418. Incubate for 2-3 days until cell death becomes apparent.

7. Once cells begin dying, change the media regularly (2 ml DMEM/5% FBS + 0.5 mg/ml G418). Keep changing media until the media is relatively clear of dead cells.

8. Isolated patches of cells should be evident, often only single cells. At this point, change the media on cells to include 10% FBS and G418. Change media every 2-3 days until foci develop.

9. Once macroscopic foci have developed, mark each with a pen. Prepare cloning rings by cutting off the large end of 200 μl pipett tips, leaving 0.5 cm of wide end. Discard long thin end. Autoclave.

10. Once wells containing well-isolated foci have been identified, aspirate off media from one well at a time and rinse with 1-2 ml PBS, aspirate off.

11. With the sterile forceps, pick up a single cut pipet tip end from the CUT end. Gently touch the manufactured flat end into the sterile vacuum grease, being sure the entire rim of the tip is covered. However, do not use too much as the grease is toxic to cells. Now gently place the greased end over the foci, avoiding contact with the cells. Gently tap the pipet tip down as to form a seal.

12. Add 100 μl 0.025% trypsin solution to the chamber. Be careful to observe if the chamber is leaking, and incubate with trypsin for 60 seconds. At this point, aspirate the trypsin solution up and down several times in the chamber and add the entire 100 μl to a fresh 2 ml well. Use 100 μl of media from the new well to rinse out the cloning chamber. Continue now to the next well, trying to get 24-30 clones from the 36 wells, and grow cells in DMEM/10% FBS + 0.5 mg/ml G418 until dense.

13. Once cells have covered most of the small well, split cells to larger plates, leaving a few cells in the small well for stock. Grow these plates until dense.

14. Screen for clones for expression of α_2 adrenergic receptors: Rinse plates with PBS, and scrape into ~1 ml of TME buffer. In a small tube, sonicate cell briefly to disrupt, and perform radioligand binding using 10-12 nM rauwolscine +10 μM phentolamine for non-specific.

APPENDIX D

[^{3}H]cAMP ASSAY PROTOCOL FOR STABLY-TRANSFECTED JEG-3 CELLS

Reagents:

Tris/EDTA Buffer:	50 mM Tris-HCl 4 mM EDTA pH 7.5 at 25°C
Tris/EDTA/0.1% BSA:	100 ml Tris/EDTA (above) 100 mg BSA
Protein kinase A	Purchased from Sigma (#P-5511). Prepare 0.06 mg/ml protein kinase in Tris-EDTA-BSA buffer.
cAMP Standard:	Purchased from Sigma (#A6885) MW = 351.2 g/mole Prepare 9 mg cAMP/10 ml Tris/EDTA = 2.56 mM Dilute 1:1000 for 128 pmol/50 µl standard.
[^{3}H]cAMP:	Purchased from NEN (#NET-275) (30-50 Ci/mmole, 1 mCi/ml) Dilute to 0.9 pmol/50 µl in Tris/EDTA buffer

3-Isobutyl-1-methylxanthine (IBMX): Purchased from Sigma (#I-5879).

Drug incubation and cell lysis:

1. Plate out cells in 24-well format two days before assay, typically oneconfluent 10 cm plate will provide enough cells for two or three 24-well plates. Trypsinize cells and resuspend in 5-6 ml of DMEM+FCS. Dilute 2 ml of cell suspension to 28-30 ml (for one 24 well plate). Add one ml of suspension to each well.

2. After 24 hours, change media on cells to serum-free media (1 mlwell).

3. Prepare DMEM containing 0.1 mg/ml IBMX. Stir for 20-30 minutes to dissolve, maintain at 37°C.

4. Prepare drug solutions at 5x concentration in DMEM/IBMX

5. Remove cells from incubator and rinse each well with 500 µl of serum-free media. Place cells in 37°C. water bath so that wells are just slightly immersed.

6. Aspirate rinse media and feed cells 400 µl of DMEM/IBMX. Preincubate cells for two minutes with IBMX.

7. Following pre-incubation, add 100 µl of 5x drug solution (or IBMX media for basal). Incubate with drugs for 15 minutes at 37°C.

8. Following incubation, aspirate off medium and add 150 µl of cold Tris/EDTA buffer. Place cells on ice.

9. Scrape cells into Tris/EDTA buffer and transfer to labelled microcentrifuge tubes. Cap and boil for 10 minutes. Remove from water bath and place on ice. Briefly centrifuge tubes (10-20 seconds) to spin down condensation. Samples may be frozen at this time.

[^{3}H]cAMP assay procedure:

1. Label two sets of microcentrifuge tubes for procedure. First prepare duplicate tubes labelled A-L for standard curve. Also label one set of 10 tubes for serial 1:1 dilution of the 128 pmol cAMP standard. Dilute 200 µl of 128 pmol standard 1:1 with 200 µl Tris/EDTA buffer. Continue with 1:1 dilutions down to 0.125 pmol/50 µl. Add 50 µl of dilution to assay tubes on ice.

2. Centrifuge cytosol samples in microfuge and transfer 50 µl of cytosol to numbered microcentrifuge tubes on ice.

3. Add 50 µl of [^{3}H]cAMP to each sample. Also add 50 µl of [^{3}H]cAMP to scintillation vials for direct counts.

4. Add 100 µl of cold protein kinase A to tubes (except L). Tube L receives no protein kinase, but instead 100 µl of 0.1 % BSA/Tris solution. Cap, vortex and place on ice in refrigerator. Incubate for two hours.

5. During incubation, prepare 2% BSA/charcoal solution.

400 mg BSA
20 ml Tris/EDTA buffer
Allow 1 hour to dissolve
Add 520 mg activated charcoal, stir gently

6. Following 2 hour incubation, uncap tubes and add 100 µl of BSA/ charcoal solution Vortex samples with charcoal and centrifuge for one minute. Place samples back on ice and transfer 200 µl of supernatant to scintillation vials for counting.

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