REGULATION OF ALPHA$_{2a}$-ADRENERGIC RECEPTOR EXPRESSION IN CULTURED RAT ASTROGLIA

By

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This work is dedicated to the memory of my parents, Allen and Janet Reutter.
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REGULATION OF ALPHA_2A-ADRENERGIC RECEPTOR EXPRESSION
IN CULTURED RAT ASTROGLIA

By

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Epinephrine (Epi) mediates various physiological effects via \( \alpha_{2A} \)-adrenergic receptors (\( \alpha_{2A} \)-AR). Studies in mice with a point-mutation in the gene for \( \alpha_{2A} \)-AR have shown that these receptors are responsible for the centrally-mediated depressor effects of \( \alpha_2 \)-AR agonists. These studies underscore the importance of understanding the basic cellular mechanisms involved in the expression of \( \alpha_{2A} \)-AR, of which little is known. In this study we have utilized astroglia cultured from the hypothalamus and brain stem of adult Sprague-Dawley rats as a model system in which to study factors which regulate \( \alpha_{2A} \)-AR expression. These cells contain a homogeneous population of \( \alpha_{2A} \)-AR.

We initially investigated regulation of \( \alpha_{2A} \)-AR mRNA in cultured astroglia as a result of increases in intracellular cAMP accumulation and by protein kinase C (PKC) activation. Treatment of astroglial cultures with forskolin (FSK), an adenylyl cyclase
activator, or with phorbol 12-myristate 13-acetate (PMA), a PKC agonist, caused time-
and concentration-dependent decreases in the levels of a ~4.0 kb $\alpha_{2A}$-AR mRNA
transcript. These results were mimicked by treatment of the cells with cAMP analogues or
agents which activate PKC. The decreases in $\alpha_{2A}$-AR mRNA levels caused by FSK and
PMA treatment appear to be the result of decreases in transcription of the $\alpha_{2A}$-AR gene
and are not due to decreases in $\alpha_{2A}$-AR mRNA degradation rate. In addition, FSK and
PMA treatment of cultured astroglia leads to decreased numbers of $\alpha_{2A}$-AR. These
observations suggest that $\alpha_{2A}$-AR are regulated by cAMP and PKC.

Epi can cause accumulation of cAMP or activation of PKC by stimulating $\alpha_1$- and
$\beta$-adrenergic receptors ($\alpha_1$-AR; $\beta$-AR). Therefore we also studied the effect of Epi on
$\alpha_{2A}$-AR expression. Our studies showed that Epi elicits a dose- and time-dependent
decrease in steady state levels of $\alpha_{2A}$-AR mRNA and number of $\alpha_{2A}$-AR, effects which are
mediated by $\alpha_1$-AR and $\beta$-AR. Taken together, these results indicate that expression of
$\alpha_{2A}$-AR is regulated in a heterologous manner by Epi, via $\alpha_1$-AR and $\beta$-AR, and their
intracellular signaling pathways.
CHAPTER 1
INTRODUCTION

The catecholamines epinephrine (Epi) and norepinephrine (NE) mediate a variety of physiological responses via several subtypes of adrenergic receptors. These catecholamines play a role in control of functions such as glycogenolysis, lipolysis, platelet aggregation, vasoconstriction, myocardial contractile force and rate, and bronchodilation. Within the central nervous system (CNS) Epi and NE act as neurotransmitters and contribute to control of sympathetic nervous system outflow, state of wakefulness and attention, modulation of pain afferents, and control of feeding behavior. In addition, perturbations of catecholaminergic systems may play a role in pathological conditions such as hypertension, pheochromocytoma, multiple system atrophy with sympathetic nervous system degeneration, obesity, Parkinson's disease, Alzheimer's disease, chronic pain, and addiction. Investigators have tried since the turn of the century to elucidate the mechanisms by which Epi and NE exert their effect on responsive tissue, and to understand how adrenergic receptors are involved in those responses.
Characterization of Alpha-2 Adrenergic Receptors

Evidence for Multiple Alpha Adrenergic Receptor Subtypes

The receptor concept was initially proposed in 1878 as an explanation for the effect of certain drugs on salivary flow (Langley, 1878). This concept was extended to suggest that Epi and NE acted at more that one “receptive mechanism,” because ergot alkaloids produced differential blockade of catecholamine-induced effects (Dale, 1906). This ultimately led to the hypothesis that cellular responsiveness was proportional to the number of receptors occupied by the catecholamine (Clark, 1937). Then, in 1948, Ahlquist proposed that the effects of catecholamines were mediated by two different adrenergic receptors, which he termed alpha and beta. He based his conclusions on observations that responses in a variety of tissues fit into two groups based on the rank-order potency of a series of structurally related catecholamines. Those responses insensitive to blockade by ergot alkaloids were mediated by beta-adrenergic receptors (β-AR), while those sensitive to blockade were mediated by alpha-adrenergic receptors (α-AR). These studies provided the basis for further studies to delineate the pharmacology, and eventually, the molecular biology of adrenergic receptors.

Further evidence for the pharmacological classification of adrenergic receptors as alpha and beta was provided with antagonists which blocked certain responses. Dichloroisoprenaline was the first agent found to block responses associated with β-AR, but not α-AR (Powell and Slater, 1957; Moran and Perkins, 1958). In addition the α-AR antagonists phentolamine and phenoxybenzamine increased NE overflow resulting from
nerve stimulation (Brown and Gillespie, 1957). These α-AR antagonists were initially thought to inhibit neuronal uptake of NE, resulting in increased overflow of NE after nerve stimulation (Thoenen et al., 1964; Langer, 1970). However, Langer (1970) also showed that phenoxybenzamine increased the amount of NE released per stimulus. The stimulus-dependent increase in NE release caused by phenoxybenzamine was also coupled to an increase in release of dopamine β-hydroxylase (DePotter et al., 1971; Johnson et al., 1971), an enzyme which catalyzes the synthesis of NE from dopamine and is coreleased with NE. These results were confirmed for both phenoxybenzamine and phentolamine at concentrations which did not inhibit reuptake, but enhanced neurotransmitter overflow elicited by nerve stimulation (Starke et al., 1971). These results suggested that α-AR antagonists caused an increase in neurotransmitter release without effecting reuptake.

In contrast to α-AR antagonists, α-AR agonists were shown to decrease neurotransmitter release. Clonidine, an α-AR agonist, was found to decrease NE release (Anden et al., 1970). Because NE is also an α-AR agonist, these results suggested that NE may act to inhibit its own release. This led to the concept of presynaptic regulation of NE release (Farnebo and Hamberger, 1971; Kirpekar and Puig, 1971; Langer et al., 1971; Starke, 1971). A presynaptic α-AR was believed to mediate a feedback mechanism whereby NE could act to inhibit its own release (Kirpekar and Puig, 1971).

The pharmacology of α-AR had previously been shown to differ among different tissues suggesting the tissues may express different α-AR. The initial hypothesis that multiple α-AR existed was based on observations that different structural requirements were needed for antagonists and agonists to interact with α-AR in the rat vas deferens and
rabbit intestine (van Rossum, 1965). Delbare and Schmitt (1973) suggested that the differing structural requirements for drug interaction were due to different receptors, which they termed $\alpha_1$-AR and $\alpha_2$-AR by analogy with the earlier named $\beta_1$-AR and $\beta_2$-AR (Lands et al., 1967). Pharmacological studies utilizing phenoxybenzamine (Dubocovich and Langer, 1974) or clonidine (Starke et al., 1974) implied that pre- and postsynaptic $\alpha$-AR existed and were also different. This resulted in the hypothesis that $\alpha_1$-AR were located postsynaptically while $\alpha_2$-AR were located presynaptically (Langer, 1974).

Subsequently, Berthelsen and Pettinger (1977) suggested that the differences between $\alpha_1$-AR and $\alpha_2$-AR were functional and not anatomic. They proposed that the receptor which mediated vasoconstriction be designated $\alpha_1$, while the receptor which mediated inhibition of neurotransmitter release from sympathetic nerve terminals be designated $\alpha_2$. Finally, radioligand binding assays confirmed the existence of distinct $\alpha_1$-AR and $\alpha_2$-AR based on the rank orders of potency of various agonists and antagonists at these receptor subtypes (Fain and Garcia-Sainz, 1980), and hence provided a pharmacologic definition of these receptors instead of anatomic or functional definitions.

Developing along with the concept of multiple adrenergic receptors, was the concept that the different adrenergic receptor subtypes mediated different biochemical responses in the cell. Epi was known to act at $\alpha$-AR and $\beta$-AR to inhibit and stimulate cyclic AMP synthesis, respectively (Robison et al., 1967; Turtle and Kipnis, 1967). In many tissues which contained $\alpha$-AR, cyclic AMP synthesis was not inhibited with $\alpha$-AR agonists (Robison et al., 1970), raising questions regarding the biochemical pathways activated by $\alpha$-AR. Questions regarding the biochemical pathways associated with $\alpha$-AR
stimulation were answered after division of $\alpha$-AR into $\alpha_1$-AR and $\alpha_2$-AR subtypes (Delbare and Schmitt, 1973). Stimulation of $\alpha_1$-AR resulted in phosphoinositide (PI) turnover and increased intracellular calcium ($Ca^{++}$) concentrations, while stimulation of $\alpha_2$-AR was associated with inhibition of adenylyl cyclase activity and attenuation of cAMP accumulation (Wikberg, 1979; Fain and Garcia-Sainz, 1980). The ability of $\alpha_2$-AR to inhibit basal and stimulated adenylyl cyclase activity was demonstrated in a variety of systems including: human platelets (Jakobs et al., 1976); rabbit platelets (Tsai and Lefkowitz, 1978); rat pancreatic islets (Katada and Ui, 1981); hamster adipocytes (Aktories et al., 1979); and NG108-15 cells (Sabol and Nirenberg, 1979). Thus, the existence of $\alpha_1$-AR and $\alpha_2$-AR as separate subtypes had been defined pharmacologically and biochemically.

Evidence for Multiple Alpha-2 Adrenergic Receptor Subtypes

The initial subclassification of $\alpha_2$-AR was based on pharmacological studies which showed the differential ability of the $\alpha_1$-AR antagonist prazosin to inhibit binding of the specific $\alpha_2$-AR antagonists $[^3H]$-yohimbine and $[^3H]$-rauwolscine to a variety of tissues and cell lines. Prazosin bound to the platelet $\alpha_2$-AR with low affinity ($K_i = 200-300$ nM) and to the $\alpha_2$-AR from neonatal rat lung with high affinity ($K_i = 5-10$ nM; Bylund, 1985; Nahorski et al., 1985). Based on these differences two laboratories independently identified $\alpha_2$-AR in the human platelet and neonatal rat lung as $\alpha_{2A}$-AR and $\alpha_{2B}$-AR, respectively (Bylund, 1985; Nahorski et al., 1985). This was followed by evidence that the HT29 cell line contained only $\alpha_{2A}$-AR, while the NG108-15 cell line contained only
\( \alpha_2b\text{-AR} \) (Bylund et al., 1988). While \( \alpha_2\text{-AR} \) were initially subclassified based on differential abilities of antagonists to inhibit radioligand binding, functional studies confirmed that both subtypes were able to inhibit cAMP synthesis (Bylund and Ray-Prenger, 1989).

The \( \alpha_2\text{-AR} \) in the opossum kidney (OK) cell line showed a pharmacological profile intermediate between the \( \alpha_{2A}\text{-AR} \) and \( \alpha_{2b}\text{-AR} \) suggesting a third subtype of \( \alpha_2\text{-AR} \) (Murphy and Bylund, 1988). This receptor bound prazosin with an affinity similar to the \( \alpha_{2b} \) subtype, but bound rauwolscine with a higher affinity. Further studies demonstrated that the \( \alpha_2\text{-AR} \) in the OK cell line and in OK tissue was a third subtype, which was named the \( \alpha_{2c}\text{-AR} \) (Blaxall et al., 1991). This subtype was also shown to exist in the human retinoblastoma cell line, Y79 (Gleason and Hieble, 1992).

Studies in the bovine pineal, rat submaxillary gland and in a rat pancreatic islet tumor cell line, RINm5F, suggested the possibility of a fourth \( \alpha_2\text{-AR} \) subtype (Simonneaux et al., 1991; Michel et al., 1989b; Remaury and Paris, 1992). Like the \( \alpha_{2A}\text{-AR} \) this subtype (named \( \alpha_{2D}\text{-AR} \)) had low affinity for prazosin, but unlike the \( \alpha_{2A}\text{-AR} \), it had low affinity for rauwolscine and yohimbine. However, subclassification of this receptor was controversial. Some radioligand binding studies in the rat CNS referred to this receptor as \( \alpha_{2D} \) (MacKinnon et al., 1992), while others referred to it as \( \alpha_{2A} \) (Uhlén et al., 1992). Molecular cloning studies later showed that this receptor is a species homologue of the human \( \alpha_{2A}\text{-AR} \) and is not a distinct subtype (Link et al., 1992).

The first \( \alpha_2\text{-AR} \) cloned was the \( \alpha_{2A}\text{-AR} \) from human platelets (Kobilka et al., 1987b). Subsequently, genes for the human \( \alpha_{2b}\text{-AR} \) and \( \alpha_{2c}\text{-AR} \) were identified (Regan et
al., 1988; Lomasney et al., 1990). These clones were identified as $\alpha_2$C10, $\alpha_2$C2 and $\alpha_2$C4 ($\alpha_{2A}$-AR, $\alpha_{2B}$-AR, $\alpha_{2C}$-AR, respectively) based on their chromosomal locations (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990). Similarly, three genes for $\alpha_2$-AR have been identified in the rat (Voigt et al., 1991; Lanier et al., 1991; Chalberg et al., 1990; Zeng et al., 1990) and in the mouse (Chruscinski et al., 1992; Link et al., 1992). The pharmacology of the cloned $\alpha_2$-AR closely matched the pharmacology of $\alpha_2$-AR subtypes identified in tissues and cell lines (Bylund et al., 1994). The mouse and rat homologues of the human $\alpha_{2B}$-AR and $\alpha_{2C}$-AR have pharmacology similar to the human clones, but the $\alpha_{2A}$-AR differs in that the mouse and rat clones have low affinity for yohimbine and rauwolscine compared to the human clone. This difference in pharmacology appears to be due to a single amino acid substitution in the mouse $\alpha_{2A}$-AR (Link et al., 1992).

Because only three genes encode for $\alpha_2$-AR in the human, rat and mouse, the designation of subtypes across species as $\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$ appears to be appropriate. The subtype originally identified pharmacologically as $\alpha_{2D}$ (Michel et al., 1989) appears to be a species homologue of the human $\alpha_{2A}$-AR, based on cloning studies (Link et al., 1992). Therefore, throughout this manuscript the rat $\alpha_2$-AR will be referred to as $\alpha_{2A}$-AR, $\alpha_{2B}$-AR, and $\alpha_{2C}$-AR.

The existence of several subtypes of $\alpha_2$-AR implied that each subtype may mediate specific physiological responses. The lack of subtype-selective agonists or antagonists has precluded the pharmacological study of subtype-specific responses. Immunohistochemical and in situ hybridization techniques, however, have been used to identify cellular and
tissue localization of α₂-AR subtypes, and have led to hypotheses for the physiological role of each of the subtypes. Within the CNS all three subtypes have been identified by in situ hybridization. Messenger RNA for α₂B-AR has been detected in the thalamic nuclei, but not in the rest of the CNS (Nicholas et al., 1993; Scheinin et al., 1994). Expression of α₂A-AR and α₂C-AR in the CNS is more widespread. α₂C-AR mRNA has been identified in the basal ganglia, olfactory tubercle, hippocampus, and cerebral cortex, while α₂A-AR mRNA has been detected in the cerebral cortex, septum, hypothalamus, hippocampus and amygdala (Nicholas et al., 1993; Scheinin et al., 1994). Within the largest noradrenergic cell group in the brain, the locus ceruleus, only α₂A-AR have been identified (Nicholas et al., 1993; Scheinin et al., 1994). This location implies that α₂A-AR are involved in mediating decreases in sympathetic nervous system outflow (Correa-Sales et al., 1992). In addition, α₂A-AR mRNA is expressed in CNS regions involved in cardiovascular control such as the nucleus tractus solitarius, rostral ventrolateral medulla and the intermediolateral cell column (Nicholas et al., 1993; Scheinin et al., 1994). Messenger RNA for α₂A-AR is also present in the dorsal root ganglia and spinal cord (Lawhead et al., 1992; Nicholas et al., 1993; Stafford-Smith et al., 1994), suggesting that α₂A-AR are involved in the antinociceptive effects of α₂-AR agonists. Immunohistochemical studies have found that α₂A-AR are located in brainstem and spinal cord regions involved in autonomic functions (Rosin et al., 1993), lending additional evidence to the hypothesis that α₂A-AR are involved in cardiovascular control.

Recently, studies in mice with mutations or knockouts of α₂-AR genes have provided additional evidence for the subtype-specific role of α₂-AR in cardiovascular
function. In studies of knockout mice, $\alpha_{2B}$-AR were demonstrated to be the $\alpha_2$-AR subtype on peripheral resistance vessels which mediates vasoconstriction (Link et al., 1996). Within the CNS, $\alpha_{2A}$-AR were shown to be involved in the long-lasting hypotensive response to $\alpha_2$-AR agonists (MacMillan et al., 1996).

**Regulation of $\alpha_{2A}$-AR Function and Expression**

**Terminology**

The responsiveness of cells to hormone or neurotransmitter stimulation is regulated by changes in the functional state of the receptors for the hormone or neurotransmitter, as well as the number of receptors on the cell surface. Receptor responsiveness to a stimulus has been demonstrated to decrease in the continued presence of that stimulation, a phenomenon known as desensitization. Examples of receptor desensitization include photoadaptation (Liebman and Pugh, 1980) and tolerance to pharmacologic agents (Rubin and Rosen, 1975; Sibley and Lefkowitz, 1985). The widespread occurrence of desensitization across receptor types suggests its importance in cellular processes. In addition, desensitization appears to act as an intracellular feedback phenomenon turning off cellular response to a single stimulus or reducing responsiveness in general to other stimuli.

Desensitization of G-protein coupled receptors refers to a variety of processes which render the receptor incapable of interacting with its G-protein, resulting in decreased cellular responsiveness to hormones or neurotransmitters. For the purpose of
this manuscript, time-dependent processes involved in desensitization will be defined as follows:

Uncoupling - rapid (seconds to minutes) impairment of the ability of the receptor to interact with its G-protein (Liggett et al., 1992; Hausdorff et al., 1990; Dohlman et al., 1991)

Sequestration - movement of the receptor away from the cell surface to an intracellular location over a time frame of minutes to hours (Perkins et al., 1991)

Downregulation - the long-term (hours to days) decrease in receptor number as a result of decreased receptor synthesis or increased receptor degradation (Bouvier et al., 1989; Collins et al., 1990; Lohse, 1993)

In addition, receptor desensitization can be divided based on the causative stimulus:

Homologous - result of agonist binding to its receptor

Heterologous - result of activation of intracellular pathways by other ligands and does not require agonist occupancy of the receptor

The causative and time-dependent processes of receptor desensitization are complementary, but very different. For example, agonist-dependent uncoupling of the β-AR as a result of β-AR kinase (βARK) phosphorylation is rapid, homologous and results in loss of receptor function (Benovic et al., 1990, 1991). In contrast, the cAMP-dependent downregulation of β-AR is slow, heterologous and results in decreased receptor number (Bouvier et al., 1989).
In addition to desensitization, G-protein-mediated pathways can be sensitized. Such sensitization appears to be the result of heterologous interaction of G-protein-mediated pathways (Hoffman et al., 1986; Hadcock et al., 1990, 1991; Sakaue and Hoffman, 1991; Morris et al, 1991). By analogy to the processes defined above for desensitization, sensitization appears to involve short-term (minutes to hours) increases in responsiveness of receptor-effector pathways, as well as long-term (hours to days) upregulation of components of receptor-effector pathways. For the purposes of this manuscript, the term “regulation” will be used to collectively refer to processes involved in the sensitization or desensitization of G-protein-coupled receptors and their signal transduction pathways. The terms “upregulation” and “downregulation” will be used to specifically refer to increases and decreases, respectively, in the amount of receptor.

One family of receptors that are regulated in this fashion are the β-AR, which transduce an Epi or NE signal into a stimulation of the intracellular enzyme adenylyl cyclase (Perkins and Moore, 1973; Tate et al., 1991). The β₂-AR subtype was the first G-protein-coupled hormone or neurotransmitter receptor purified and cloned (Dixon et al., 1986), and therefore it has been the model for studying G-protein coupled receptor regulation (Sibley and Lefkowitz, 1985). The cellular and molecular mechanisms of homologous and heterologous regulation have been largely defined for the β₂-AR (reviewed by Hadcock and Malbon, 1993; Lohse, 1993; Hein and Kobilka, 1995). The β₂-AR can be phosphorylated by βARK (Benovic et al., 1986, 1989, 1990), protein kinase A (PKA; Blake et al., 1987; Clark et al., 1989), or protein kinase C (PKC; Pitcher et al., 1992; Bouvier et al., 1987; Johnson et al., 1990), resulting in uncoupling of the receptor...
from its effector pathway. The β₂-AR can also be reversibly sequestered to intracellular compartments as a result of agonist binding (vonZastrow and Kobilka, 1992, 1994; vonZastrow et al., 1993). Finally, the β₂-AR can be downregulated via agonist-dependent and independent events (Bouvier et al., 1989; Collins et al., 1990). These mechanisms are summarized in Table 1-1, and have provided the basis for studies on the regulation of other G-protein-coupled receptors.

**Table 1-1. Mechanisms of β₂-AR Regulation**

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>Uncoupling</td>
<td></td>
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<tr>
<td>βARK/β-arrestin</td>
<td>Homologous</td>
</tr>
<tr>
<td>PKA/PKC</td>
<td>Heterologous</td>
</tr>
<tr>
<td>Sequestration</td>
<td>Homologous</td>
</tr>
<tr>
<td>Downregulation</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td></td>
</tr>
<tr>
<td>Decreased Transcription</td>
<td>Heterologous</td>
</tr>
<tr>
<td>Increased mRNA Degradation</td>
<td>Heterologous</td>
</tr>
<tr>
<td>Receptor Degradation</td>
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<tr>
<td>Agonist-Specific</td>
<td>Homologous</td>
</tr>
<tr>
<td>PKA/PKC-Mediated</td>
<td>Heterologous</td>
</tr>
</tbody>
</table>

From Lohse, 1993
Homologous Regulation of $\alpha_2$-AR

Study of the cellular and molecular mechanisms involved in $\alpha_2$-AR regulation has lagged behind that of the $\beta_2$-AR. Human platelets were the first model system used for studying $\alpha_2$-AR regulation. Cooper et al. (1978) demonstrated that the platelet $\alpha_2$-AR was subject to homologous regulation, because incubation of platelets with Epi resulted in desensitization of $\alpha_2$-AR via decreased function and decreased number. This inverse relationship between increased catecholamine concentration and decreased platelet $\alpha_2$-AR function and number was shown to occur in pathophysiological conditions such as pheochromocytoma, a catecholamine-producing tumor (Davies et al., 1981; Brodde and Bock, 1984). The reverse was also shown to occur in multiple system atrophy with sympathetic nervous system degeneration where decreased catecholamine concentrations led to increased platelet $\alpha_2$-AR expression (Davies et al., 1981). $\alpha_2$-AR on platelets from dogs and rabbits were also regulated similarly (Meyers et al., 1983; Hamilton et al., 1985; Deighton et al., 1988), indicating that similar regulatory mechanisms operated across species lines. The cellular mechanisms of this regulation involved guanine nucleotides which reduced the high affinity state of the receptor, indicating involvement of a guanine nucleotide binding protein (Michel et al., 1980; Schloos et al., 1987). In addition, phosphorylation of the inhibitory G protein (G_i) by protein kinase C (PKC) resulted in suppression of the inhibitory adenylyl cyclase pathway associated with $\alpha_2$-AR in human platelets (Jakobs et al., 1985; Katada et al., 1985; Watanabe et al., 1985), suggesting that the $\alpha_2$-AR signaling pathway was also subject to heterologous regulation (see later...
The $\alpha_2$-AR on platelets was subsequently cloned and was classified as the $\alpha_{2A}$ subtype (Kobilka et al., 1987). Like the $\beta_2$-AR, the $\alpha_{2A}$-AR have become the model for study of $\alpha_2$-AR regulation.

Additional information regarding homologous regulation of $\alpha_2$-AR has come from the use of cell lines and transfected cells as model systems. The $\alpha_{2A}$-AR is coupled to inhibition of adenylyl cyclase in virtually every system studied. Therefore, modulation of cellular cAMP levels is generally used as a measure of the functional state of $\alpha_{2A}$-AR. In the human colonic adenocarcinoma cell line HT29, activation of $\alpha_{2A}$-AR results in inhibition of adenylyl cyclase activity (Turner et al., 1985), a response which is desensitized in the continued presence of $\alpha_2$-AR agonists (Jones et al., 1987; Jones and Bylund, 1988). This response is notable within 5 min of agonist exposure and is readily reversible, even after 60 min of NE incubation (Jones et al., 1990). However, after 18 h of NE incubation, $\alpha_{2A}$-AR receptor numbers decreased (Jones et al., 1990). Similarly, experiments in transfected Chinese hamster ovary (CHO) cells demonstrated that agonist exposure resulted in decreased high-affinity binding (Eason and Liggett, 1992), which is suggestive of uncoupling of the $\alpha_{2A}$-AR from its G-protein (Hausdorff et al., 1990). This was followed by sequestration of $\alpha_{2A}$-AR and a long-term decrease in the amount of Gi and $\alpha_{2A}$-AR (Eason and Liggett, 1992). These studies demonstrated that $\alpha_{2A}$-AR can be desensitized via short- and long-term changes, but did not demonstrate the mechanisms associated with those changes.

The molecular mechanisms for these changes in $\alpha_{2A}$-AR sensitivity had been proposed to include phosphorylation of the receptor by $\beta$ARK, because $\beta$ARK could
phosphorylate the purified receptor in a reconstituted system (Benovic et al., 1987). Studies in transfected Chinese hamster fibroblasts (CHF) provided the first evidence in whole cells that $\alpha_{2A}$-AR were phosphorylated by $\beta$ARK in an agonist-dependent fashion (Liggett et al., 1992). In a manner similar to the $\beta_2$-AR (Benovic et al., 1990, 1991), agonist-dependent desensitization of $\alpha_{2A}$-AR occurred rapidly after agonist exposure, was accompanied by phosphorylation of the receptor, and was dependent on agonist occupancy of the receptor (Liggett et al., 1992). These events led to sequestration of $\alpha_{2A}$-AR in CHF, but not to a long-term decrease in $\alpha_{2A}$-AR number (Liggett et al., 1992) as was seen in CHO and HT29 cells (Jones et al., 1990; Eason and Liggett, 1992). Instead, a decrease in $G_i$ was noted, further desensitizing the $\alpha_{2A}$-AR-mediated inhibition of adenylyl cyclase (Liggett et al., 1992). The different regulatory results observed in HT29 cells (Jones et al., 1990), CHO cells (Eason and Liggett, 1992) and CHF (Liggett et al., 1992) suggest that $\alpha_{2A}$-AR may be regulated in a cell- or tissue-specific manner.

**Heterologous Regulation of $\alpha_{2A}$-AR**

As mentioned previously, the platelet model provided the first evidence of heterologous regulation of the $\alpha_{2A}$-AR signaling pathway. Platelets treated with the phorbol ester, 12-$O$-tetradecanoylphorbol-13-acetate did not exhibit an Epi-induced inhibition of adenylyl cyclase activity (Jakobs et al., 1985). This effect of phorbol ester was due to activation of PKC and subsequent phosphorylation of $G_i$ (Watanabe et al., 1985; Katada et al., 1985). It appeared that the phosphorylated form of $G_i$ could not be activated by GTP and therefore could not inhibit adenylyl cyclase activity. Similarly, in
NG108-15 cells, phorbol esters decrease responsiveness of $\alpha_{2B}$-AR without changing $\alpha_{2B}$-AR number, suggesting action at the level of $G_i$ (Convents et al., 1989). This inhibitory response to phorbol esters was also shown to occur with the $G_i$-linked response to bradykinin (Convents et al., 1989), providing further evidence of the heterologous nature of PKC regulation of $G_i$-mediated pathways. PKC activation has not resulted in desensitization of $\alpha_{2A}$-AR or their associated signaling components in HT29 cells or pancreatic $\beta$-cell lines (Sakaue and Hoffman, 1991; Hamamdzic et al., 1995).

The first demonstration that $\alpha_2$-AR themselves were regulated in a heterologous fashion was made in studies with cultured astroglia. Treatment of cultured astroglia with insulin resulted in downregulation of $\alpha_2$-AR (Richards et al., 1987) although the specific $\alpha_2$-AR subtype was not known at that time. These results were unique in that they were also the first demonstration of $\alpha_2$-AR regulation in non-transfected, non-transformed cells containing transcriptional machinery. Studies in HT29 cells also showed that insulin downregulated $\alpha_{2A}$-AR, and that other growth factors produced similar decreases (Devedjian et al., 1991). The downregulatory effect of insulin and growth factors was shown to be due to decreased transcription of the $\alpha_{2A}$-AR gene resulting in decreases levels of $\alpha_{2A}$-AR mRNA (Devedjian et al., 1991).

Because of the inhibitory coupling of $\alpha_{2A}$-AR to adenylyl cyclase, the question arose of whether cAMP may regulate $\alpha_{2A}$-AR. Studies in HT29 cells showed that $\alpha_{2A}$-AR mRNA and protein levels increased after exposure of the cells to drugs which stimulate adenylyl cyclase and result in accumulation of intracellular cAMP (Sakaue and Hoffman, 1991). The increase in $\alpha_{2A}$-AR expression occurred by transiently stabilizing the $\alpha_{2A}$-AR
mRNA transcript and by increasing transcription rate (Sakaue and Hoffman, 1991). In order to elucidate the transcriptional mechanisms involved in $\alpha_{2A}$-AR expression, JEG-3 cells were transfected with a reporter gene coupled to the $\alpha_{2A}$-AR promoter. Cyclic AMP increased $\alpha_{2A}$-AR gene expression via a PKA-mediated increase in activity of the 5' promoter region of the $\alpha_{2A}$-AR gene. These results suggested the involvement of a cyclic AMP responsive element (CRE) in the promoter region. Analysis of the promoter region for the human $\alpha_{2A}$-AR did not, however, reveal the presence of a CRE (Handy et al., 1992). Similarly, a CRE was not reported for the rat $\alpha_{2A}$-AR promoter (Handy et al., 1995). It is possible that the actions of PKA are mediated by nuclear factors which are not classically associated with cyclic AMP accumulation.

Rationale

Three subtypes of $\alpha_2$-adrenergic receptors ($\alpha_2$-AR) have been identified by pharmacology and molecular cloning (Bylund et al., 1994). All three subtypes can be activated by the endogenous catecholamines epinephrine (Epi) and norepinephrine, and inhibit the accumulation of cellular cyclic AMP via inhibition of adenylyl cyclase (Limbird, 1988). The physiological function of the different subtypes appears to be determined by their pattern of cellular and tissue localizations. For example, activation of $\alpha_{2A}$-AR in the central nervous system appears to mediate decreases in blood pressure (MacMillan et al., 1996), while activation of $\alpha_{2B}$-AR on resistance vessels increases blood pressure (Link et al., 1996). The differences in cell and tissue distribution of $\alpha_2$-AR may also contribute to differing modes of regulation of expression of each subtype. Regulation of central $\alpha_{2A}$-AR
represents an important way through which adrenergic signaling is modulated. Upregulation of $\alpha_{2A}$-AR could potentiate the inhibitory actions of $\alpha_1$-AR agonists, whereas down-regulation may lead to increased activity of adrenergic signaling pathways. An understanding of the regulation of expression of $\alpha_2$-AR in a variety of cell type and tissues may lead to novel methods to affect changes in $\alpha_{2A}$-AR expression without changing the expression or function of $\alpha_{2B}$-AR or $\alpha_{2C}$-AR.

Information regarding $\alpha_{2A}$-AR regulation has come largely from the use of cell lines and transfected cells as model systems. Experiments in such transformed cells allow study of receptor regulation within a relatively homogeneous model. Astroglial cells cultured directly from rat brain (cultured astroglia) have been shown to contain a relatively homogeneous population of $\alpha_{2A}$-AR (see Chap. 3). In addition cultured astroglia contain $\beta$-AR (McCarthy, 1983; Baker et al., 1986), $\alpha_1$-AR (Hirata et al., 1983; Murphy and Pearce, 1987) and $\alpha_2$-AR (Richards et al., 1989) that are functionally coupled to signal transduction pathways which include modulation of cAMP accumulation ($\beta$-AR and $\alpha_2$-AR: Baker et al., 1986; Atkinson and Minnemann, 1991, 1992) and increases in phosphoinositide (PI) hydrolysis ($\alpha_1$-AR: Wilson and Minnemann, 1990, 1991). One of the products of PI hydrolysis is diacylglycerol which rapidly activates protein kinase C (PKC). Therefore, because cultured astroglia contain functional $\alpha_1$-AR, $\alpha_2$-AR and $\beta$-AR they provide an excellent model system in which to study regulation of $\alpha_{2A}$-AR by a variety of pathways. No studies have investigated the role of heterologous regulation of $\alpha_{2A}$-AR by pathways associated with $\alpha_1$-AR and $\beta$-AR. In addition, the regulatory mechanisms for $\alpha_{2A}$-AR on astroglia are largely unknown. Therefore in this study, I have
investigated the role of these receptors and their signaling pathways in regulating expression of $\alpha_{2A}$-AR.

My specific objectives are as follows:

I. Characterize the regulation of $\alpha_{2A}$-AR mRNA levels in astroglia by cAMP and PKC.

II. Characterize the regulation of $\alpha_{2A}$-AR mRNA levels in cultured astroglia by endogenous ligands which cause cAMP accumulation or activate PKC.

III. Investigate the role of epinephrine, cAMP accumulation and PKC activation on $\alpha_{2A}$-AR number and the ability of $\alpha_{2}$-AR agonists to inhibit cAMP production in astroglia.
Materials

Adult Sprague-Dawley rats were from our breeding colony, which originated from Charles River Farms, Wilmington, MA. Dulbecco's modified Eagles medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO, Grand Island, NY. [\(\alpha-^{32}\text{P}\)]dCTP (3,000 Ci/mmol), [\(\alpha-^{32}\text{P}\)]UTP (3,000 Ci/mmol), [\(^3\text{H}\)]-MK 912 (76 Ci/mmol), cyclic AMP radioimmunoassay kits and GeneScreen nylon membrane were purchased from Dupont-New England Nuclear, Boston, MA. Agarose and nonidet P-40 were purchased from ICN Biomedicals, Inc., Aurora, OH. Forskolin (FSK), dibutyryl cyclic AMP (db-cAMP), phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDB), mezerein (Mez), 4\(\alpha\)-phorbol, actinomycin D, (-)-epinephrine bitartrate (Epi), prazosin, yohimbine, s-(-)-propranolol, angiotensin II (Ang II), and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) were purchased from Sigma Chemical, St. Louis, MO. 1,9-Dideoxyforskolin (DDF) and H-89 were purchased from CalBiochem, La Jolla, CA. Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Sp-cAMPS), and UK 14304 were purchased from Research Biochemicals International, Natick, MA. Random Primer DNA labeling kits were purchased from GIBCO, BRL. RQ1 RNase-free DNase (1U/\(\mu\)l), proteinase K, ribonucleotides and restriction enzymes
were purchased from Promega Corp., Madison, WI. Plasmids (pGEM7) containing RG20 ($\alpha_{2A}$-AR) cDNA were kindly provided by Dr. Steven Lanier, Medical Univ. of South Carolina. Plasmids (pUC19) containing Cu-Zn superoxide dismutase (SOD) cDNA were a gift from Dr. Harry Nick, Univ. of Florida, Gainesville, FL. All other chemicals and reagents were of molecular biology grade and were purchased from Fisher Scientific, Pittsburgh, PA.

Methods

Astroglial Cultures

Astroglial cells were cultured from the hypothalamus and brain stem of adult rats as described previously (Zelezna et al., 1992). Trypsin-dissociated brain cells, suspended in DMEM containing 10% FBS, were plated in poly-L-lysine coated 100-mm culture dishes ($18 \times 10^6$ cells/dish) and were grown for 14-21 days in a humidified incubator at 37°C in 5% CO$_2$-95% air. After this time, cells were dissociated from dishes using trypsin and replated in 100-mm culture dishes for nuclear runoff and binding experiments, and in 35-mm culture dishes for RNA isolation and cyclic AMP analysis. Astroglia were grown in a humidified incubator at 37°C in 5% CO$_2$-95% air for 13-15 days before being used in experiments. At that time the cultures consisted of $>95$% astroglia, as determined by immunofluorescent staining with antibodies against glial fibrillary acidic protein (Sumners et al., 1991).
Neuronal Cultures

Neuronal cultures were prepared from whole brains of newborn Sprague-Dawley rats as described previously (Sumners et al, 1991). Trypsin-dissociated cells were resuspended in DMEM containing 10% plasma-derived horse serum (PDHS) and were plated in poly-L-lysine coated 35-mm culture dishes (3.0 × 10^6 cells per dish). Cells were grown for 3 days in a humidified incubator at 37°C in 5% CO₂-95% air, and were then treated for 2 days with 10 µM cytosine arabinoside in fresh DMEM/10% PDHS. After this, the medium with the cytosine arabinoside was removed and replaced with fresh DMEM/10% PDHS, and the cells were allowed to grow for 13-15 days before use. Such cultures consisted of approximately 90% neurons and 10% astrocytes (Sumners et al, 1991).

Drug Treatment

FSK, DDF, PMA, 4α-phorbol, Mez, PDB, and UK 14304 were dissolved in dimethyl sulfoxide (DMSO) and diluted with filter sterilized water. Sp-cAMPS, db-cAMP, yohimbine, and propranolol were dissolved in water. Prazosin was dissolved in ethanol and diluted with filter sterilized water. Epinephrine was dissolved in 10 mM ascorbate in phosphate buffered saline (PBS). Final dilutions were made into the DMEM/FBS in the culture dish. Final dilutions for the cAMP experiments were made into the DMEM without FBS in the culture dish.
Preparation of Cultured Astroglia for Light Microscopy

Cultured astroglia were prepared for light microscopy exactly as described (Ciotti et al., 1989). Cultured astroglia were treated with control vehicle (0.5% DMSO or 100 µM ascorbate in PBS), 10 µM FSK, 500 nM PMA, or 100 µM Epi for 4 or 24 h. Dishes of cultured astroglia were washed with ice-cold PBS and fixed with 2.5% glutaraldehyde in PBS for 30 min. Glutaraldehyde was then removed and dishes were washed with PBS for 2 min. PBS was then removed and the cells were covered with glia stain (0.5% Coomassie brilliant blue in 50% isopropanol containing 1% formic acid) for 1 min. Glia stain was removed and the dishes were washed twice with ice-cold PBS. Plastic coverslips were mounted with Gel/Mount.

A Zeiss Axioskop microscope with phase contrast illumination was used to observe the stained cells. Photographs were taken to document changes in cell morphology. Phase-contrast light was used for all the photographs. A Minolta X-570 camera was used with Kodak Gold 100 ISO film. Shutter speed was determined by use of the camera’s automatic control. Magnification was 200X at the film surface.

RNA Preparation and Northern Blot Analysis

Levels of α2A-AR mRNA in astroglial cultures were determined with the use of a 2.2 kb fragment of the rat RG20 (α2A-AR) cDNA. This was produced by EcoR I and Hind III digestion of pGEM7, into which the RG20 cDNA had been cloned. This fragment contained the entire RG20 coding region plus about 700 bp of the 3' untranslated
region. Levels of $\alpha_{2A}$-AR mRNA were normalized with the use of a 625 bp fragment of the rat SOD cDNA. This fragment was produced by EcoRI digestion of pUC19, into which the SOD cDNA had been cloned. The $\alpha_{2A}$-AR and SOD fragments were $^{32}$P-labeled by a random primer labeling system according to the manufacturer's directions with $[\alpha-^{32}$P]dCTP. $\alpha_{2A}$-AR and SOD mRNA was measured by Northern blot analysis of total RNA extracted from astroglial cultures. Total cellular RNA was prepared by the guanidinium-acid-phenol method (Chomczynski and Sacchi, 1987), and 20 µg aliquots were fractionated by 1% agarose-20 mM formaldehyde gel electrophoresis. Total RNA was then transferred overnight by capillary action to GeneScreen nylon membrane and crosslinked to the membrane by exposure to shortwave UV radiation (12,000 µJ/cm²) using a Stratalinker (Stratagene, La Jolla, CA). Membranes were prehybridized overnight at 45°C in hybridization buffer containing 50% formamide, 5% dextran sulfate, 5X sodium chloride-sodium phosphate-EDTA (SSPE; 1X = 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA), 5X Denhardt's reagent (1X = 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% sodium dodecyl sulfate (SDS), and 100 µg/ml denatured salmon sperm. Membranes were hybridized for 18 hours in fresh hybridization buffer (45°C) containing denatured $^{32}$P-labeled $\alpha_{2A}$-AR and SOD probes. After hybridization, membranes were washed twice (30 minutes each) in 2X SSPE/0.2% SDS at 50°C and twice (15 minutes and 5 minutes) in 0.1X SSPE at 60°C, and exposed to Kodak XAR film for 24 hours. Membranes were hybridized simultaneously with $\alpha_{2A}$-AR and SOD probes since signals for either message were no different when the probes were used simultaneously or sequentially.
Using these procedures we were able to detect an approximately 4 kb $\alpha_{2\alpha}$-AR mRNA and a 0.7 kb SOD mRNA, consistent with those seen in other studies (Lanier et al., 1991; Hamamdzic et al., 1995). Quantitation of the bands corresponding to $\alpha_{2\alpha}$-AR and SOD mRNA was achieved by arbitrary counting using a Molecular Dynamics Phosphorimaging System. Normalization of the $\alpha_{2\alpha}$-AR mRNA data was accomplished by dividing the values obtained for each $\alpha_{2\alpha}$-AR band by the value for the corresponding SOD band. All $\alpha_{2\alpha}$-AR mRNA data that are presented were normalized against SOD and expressed as a percent of control values.

**Nuclear Runoff Transcription Assay**

Astroglial cultures were treated with 10 µM FSK, 500 nM PMA or control solution (0.5% DMSO) for 1, 4, or 24 hours at 37°C. Nuclei were then isolated from two 100-mm culture dishes from each treatment group as described previously (Greenberg and Ziff, 1984). Dishes were washed twice in cold phosphate-buffered saline, astroglia were removed with the aid of a rubber policeman, and pelleted at 500 g for 5 min. The pellet was resuspended in 4 ml NP40 lysis buffer (10 mM tris-HCl pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 0.5% nonidet P-40), incubated on ice for 5 min and centrifuged at 500 g for 5 min. The nuclear pellet was washed with NP40 lysis buffer and centrifuged at 500 g for 5 min. The nuclei were resuspended in storage buffer (50 mM tris-HCl pH 8.0, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT), frozen in an acetone/dry ice bath and stored at -80°C.
The runoff transcription was performed as described (Greenberg, 1988, Celano, 1989) with some modifications. Frozen nuclei (2.5-3 \times 10^6/100 \mu l) were thawed on ice in the presence of 100 \mu l reaction buffer (10 mM tris-HCl pH 8.0, 5 mM MgCl₂, 300 mM KCl, 5 mM DTT, 1 mM each ATP, CTP, GTP) and 10 \mu l [\alpha^{-32}P]UTP (3,000 Ci/mmol). The nuclei were incubated for 15 min at room temperature. DNA was digested by addition of 20U RNase-free DNase and 12 \mu l 20 mM CaCl₂ and incubated for 5 min at room temperature. Protein degradation was initiated by incubating the mixture for 30 min at 37°C with 27 \mu l SET (5% SDS, 50 mM EDTA, 10 mM tris-HCl pH 7.4) and 2 \mu l 10 mg/ml proteinase K. Yeast tRNA (5 \mu l of 10 mg/ml) was added and the newly transcribed, labeled RNA was extracted by the guanidinium-acid-phenol method (Chomczynski and Sacchi, 1987). The radiolabeled RNA was dissolved in TES solution (10 mM TES pH 7.4, 10 mM EDTA, 0.2% SDS) to 2-3 \times 10^6 cpm/ml within each experiment, and was mixed with an equal volume of TES solution containing 600 mM NaCl (final concentration 300 mM). Two ml of the combined solution was hybridized at 65°C for 36 hr with 5 \mu g of pGEM7 plasmid immobilized to a nylon membrane (as a control) and to 5 \mu g immobilized plasmid containing inserts of the RG20 cDNA or SOD cDNA. The membranes were washed twice (15 min each) in 2X SSC/0.2% SDS (1X SSC = 150 mM NaCl, 15 mM sodium citrate pH 7.0) at 65°C and once (5 min) in 0.1X SSC at 65°C and were then exposed to Kodak XAR film with intensifying screens at -80°C for 2 weeks.
**α₂A-AR mRNA Stability**

The stability of α₂A-AR mRNA in control, FSK-treated and PMA-treated astroglia was measured by incubating astroglia in 10 µg/ml actinomycin D to block transcription. Astroglia were collected at various times after FSK or PMA treatment, total cellular RNA was extracted, and α₂A-AR mRNA levels were assessed by Northern blot analysis as described above. The α₂A-AR mRNA degradation rate (half-life) was calculated after quantitation of the bands as described above using a Molecular Dynamics Phosphorimaging System.

**Binding Studies**

Membranes from astroglial cultures were prepared as described (Richards et al., 1989). Astroglia in 100 mm culture dishes were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.2) and removed from the dishes with the aid of a rubber policeman. Cells were centrifuged at 500 g for 5 min at 4°C and the supernatant discarded. The pellet was resuspended in ice-cold PBS, frozen in an acetone/dry ice bath and stored at -80°C.

On the day of the experiment, the cells were thawed on ice and resuspended in 3 ml of ice-cold binding buffer (33 mM tris-Cl, pH 7.5, 1.0 mM EDTA, 0.1% ascorbic acid). They were then homogenized using a Tekmar Tissuemizer (setting 50 for 30 s) and centrifuged at 500 g for 5 min at 4°C. The supernatant was centrifuged at 50,000 g for 10 min at 4°C. The resulting pellet was resuspended in 500 µl binding buffer containing 140
mM NaCl and the protein content of an aliquot determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The membrane preparation was kept on ice until the binding assay was performed.

Radioligand binding was performed essentially as described (Uhlén and Wikberg, 1991) by incubating 10 µg of the membrane protein in 500 µl binding buffer containing 140 mM NaCl and [³H]-MK 912 for one hour at 25°C. Nonspecific binding was determined in the presence of 100 µM Epi. The assay was terminated by addition of 4 ml ice-cold binding buffer with 140 mM NaCl and rapid filtration through Whatman GF/B filter paper using a cell harvester (Brandel, Gaithersburg, MD). The membranes were rapidly washed three times with 3 ml ice-cold binding buffer containing 140 mM NaCl. The filters were transferred to scintillation vials containing 5 ml Liquiscint and counted in an LKB RackBeta liquid scintillation counter at an efficiency of approximately 50% for ³H. All assays were performed in triplicate.

**Cyclic AMP Extraction and Analysis**

Thirty minutes prior to treatment, growth media were removed from the astroglial cultures and replaced with fresh DMEM without FBS. Groups of 3 dishes were then treated with the appropriate drugs or controls for various times by adding the solutions to the media in the dishes. In experiments utilizing isobutyl methyl xanthine (IBMX), the serum-free media were removed by aspiration and replaced with DMEM containing 2 mM IBMX for 2 minutes before addition of FSK or PMA. In the experiments using clonidine, the growth media were removed by aspiration and replaced with serum-free DMEM with
various concentrations of clonidine (0-10 µM) for 2 min before addition of FSK or control solution (0.5% DMSO). All treatments were stopped by rapid aspiration of the media followed by the addition of 0.5 ml 6% trichloroacetic acid (TCA) to each dish.

Extraction of cellular cyclic AMP was performed as previously described (Scammell et al. 1995). The cells plus 6% TCA were removed from the dish with the aid of a rubber policeman, transferred to glass tubes and centrifuged at 2,500 g at 4°C for 15 minutes. The resulting pellets were dissolved in 1 ml of 1.0 M NaOH for protein determination (Lowry et al., 1951). The supernatants were each extracted four times with a total of 10 ml water-saturated ethyl ether. The ether phase was discarded and the samples were evaporated to dryness using a SAVANT evaporator-concentrator and then reconstituted in 0.5 ml 50 mM sodium acetate buffer (pH 6.2). Samples were stored frozen at -20°C until assayed for cyclic AMP.

The levels of cyclic AMP in cell samples were analyzed in duplicate by [125I]-cyclic AMP radioimmunoassay kit according to the directions of the kit manufacturer (Dupont, NEN). Samples were counted in a Beckman 5500 gamma counter. Sample values were obtained by interpolation from the standard curve, corrected for the dilution of the original sample, and expressed as pmol cyclic AMP per mg protein. The cross-reactivity of the antibody with cyclic GMP and ATP was less than 0.01% each.

Statistics

Each data point was obtained from four 35-mm culture dishes for the mRNA experiments and three 35-mm culture dishes for the cyclic AMP experiments. Data points
for the binding experiments were obtained from triplicate samples. Each experiment was repeated at least three times. The data on all graphs and tables are presented as mean ± SEM of the number of experiments indicated in the legend. Comparison between groups was made with the use of ANOVA followed by the Newman-Keuls test to assess statistical significance of the individual means. The results were considered statistically significant at p < 0.05. Statistical analyses were performed using Sigma Stat software (Jandel, San Rafael, CA).
CHAPTER 3
REGULATION OF $\alpha_{2A}$-ADRENERGIC RECEPTOR mRNA IN CULTURED RAT
ASTROGLIA: ROLE OF CYCLIC AMP AND PROTEIN KINASE C

Introduction

The expression and function of G-protein coupled receptors are highly regulated. The mechanisms involved in this regulation are complex and are not well known for most G-protein coupled receptors. The $\beta_2$-AR have been the model for study of G-protein coupled receptors and their regulation (Sibley and Lefkowitz, 1985; Dohlman et al., 1991), and have provided the basis for studies of other receptors. The regulation of $\beta_2$-AR can occur over time-frames of seconds to days. The short-term (seconds to minutes) changes in receptor function are typified by phosphorylation of the receptor and uncoupling from its signal transduction pathways (Hausdorff et al., 1990). In contrast, long-term (hours to days) regulation involves changes in the steady-state levels of receptor mRNA and protein (Bouvier et al., 1989). Expression and function of $\beta_2$-AR can be modulated by stimulation of $\beta_2$-AR themselves, or by stimulation of other G-protein coupled receptors, tyrosine kinase receptors or steroid receptors (reviewed by Lohse, 1993).

Like the $\beta_2$-AR, the mechanisms involved in regulation of $\alpha_{2A}$-AR expression and functional coupling to inhibition of adenylyl cyclase are complex. However, relatively
little is known about the homologous regulation of $\alpha_{2A}$-AR. The agonist-occupied $\alpha_{2A}$-AR is a substrate for phosphorylation by $\beta$ARK (Benovic et al., 1987; Liggett et al., 1992; Kurose and Lefkowitz, 1994), and therefore is subject to homologous regulation at the receptor level. Long-term agonist exposure causes a decrease in $\alpha_{2A}$-AR number (Jones et al., 1990; Eason and Liggett, 1992).

The $\alpha_{2A}$-AR gene has been reported to be subject to heterologous regulation. This regulation appears to vary depending on the cell model used in the study. Insulin and growth factors were shown to decrease transcription of $\alpha_{2A}$-AR mRNA in the HT29 cell line (Devedjian et al., 1991), but were without effect in pancreatic $\beta$-cell lines from the rat or hamster (Hamamdziec et al., 1995). Glucocorticoids did not change expression of $\alpha_{2A}$-AR in HT29 cells, but increased steady-state levels of mRNA in pancreatic $\beta$-cell lines (Hamamdziec et al., 1995). Activation of PKC with phorbol esters did not effect levels of $\alpha_{2A}$-AR mRNA in HT29 cells (Sakaue and Hoffman, 1991) or in pancreatic $\beta$-cell lines (Hamamdziec et al., 1995). PKA activation as a result of increased cAMP accumulation led to increased expression of the $\alpha_{2A}$-AR gene in HT29 cells (Sakaue and Hoffman, 1991). Taken together, these results suggest that $\alpha_{2A}$-AR gene expression may be regulated in a manner specific to the cell line used in the study. However, regulation of $\alpha_{2A}$-AR in cell lines may not be representative of regulation in non-transformed, non-transfected cells. Studies of $\alpha_{2A}$-AR regulation in non-transfected, non-transformed cells, however, are limited.

Therefore, in the present study we have used astroglial cells cultured directly from adult rat brain as a model system to study $\alpha_{2A}$-AR regulation. These non-transformed cells
have been shown to contain high levels of $\alpha_2$-AR protein (Richards et al. 1989) and $\alpha_{2A}$-AR mRNA, with very little or no $\alpha_{2C}$-AR mRNA. Our studies indicate that both increases in cellular cyclic AMP levels and PKC activation decrease steady state levels of $\alpha_{2A}$-AR mRNA via decreased transcription of the $\alpha_{2A}$-AR gene.

**Results**

Northern analysis of total RNA from astroglial and neuronal cultures and from various rat brain regions revealed a ~4.0 kb transcript that hybridized specifically with the $\alpha_{2A}$-AR (RG20) cDNA probe, indicating that this probe recognized the same transcript in the cultures and in the brain regions studied (Fig. 3-1). The mRNA detected in the present study was of similar size and distribution as the transcript reported in other studies (Lanier et al, 1991; Zeng and Lynch, 1991; Hamamdzic et al., 1995). For example, RNA isolated from cortex, hypothalamus, and brain stem appeared to contain more $\alpha_{2A}$-AR mRNA than striatum or cerebellum. This is in good agreement with previous studies which found that $\alpha_{2C}$-AR, rather than $\alpha_{2A}$-AR, predominate in the striatum and cerebellum (Zeng and Lynch, 1991; Nicholas et al, 1993). When northern blots of total RNA from neuronal cultures were hybridized with an $\alpha_{2C}$-AR probe (RG10; Lanier et al, 1991) a transcript of much smaller size (~2.5 kb) was recognized (data not shown). In addition, when northern blots of total RNA from cultured astroglia were hybridized with the RG10 probe, no transcript was recognized (data not shown), suggesting that these cultures contain no $\alpha_{2C}$-AR. Overall, these data indicate that the RG20 probe used here specifically recognizes the $\alpha_{2A}$-AR subtype.
Effects of Forskolin on $\alpha_{2A}$-AR mRNA Levels

Previous studies showed that cyclic AMP regulates $\alpha_{2A}$-AR mRNA levels in HT29 cells (Sakaue and Hoffman, 1991). Therefore, in the first series of experiments, we investigated the effects of an adenylyl cyclase activator forskolin (FSK) on steady state $\alpha_{2A}$-AR mRNA levels in astroglia as a function of incubation time and concentration. Treatment of astroglial cultures with FSK resulted in a time-dependent decrease in steady state $\alpha_{2A}$-AR mRNA levels (Fig. 3-2). Reduction of $\alpha_{2A}$-AR mRNA levels was apparent after 1 hour and was significantly different from control at 2 hours. A 90% decrease in the $\alpha_{2A}$-AR mRNA was seen within 3 hours. Levels of $\alpha_{2A}$-AR mRNA gradually increased toward 50% of control values at 48 and 72 hours. The FSK-induced decrease in $\alpha_{2A}$-AR mRNA level was also concentration dependent (Fig. 3-3). A trend toward reduction of $\alpha_{2A}$-AR mRNA levels was seen at 100 nM FSK after 4 hours, while 1 µM FSK significantly reduced the levels of this message to about 20% of control. The FSK analog DDF (10 µM), which does not activate adenylyl cyclase did not induce any change in steady state $\alpha_{2A}$-AR mRNA levels (Fig. 3-3A).

FSK has been shown to stimulate adenylyl cyclase resulting in an increase in intracellular cyclic AMP. Therefore, in the next set of experiments, we assessed the effect of two membrane permeable, non-hydrolyzable cyclic AMP analogs on steady state $\alpha_{2A}$-AR mRNA levels in order to indicate whether the action of FSK on $\alpha_{2A}$-AR mRNA levels was mediated by increased cyclic AMP levels. Figure 3-4 shows that both db-cAMP and Sp-cAMPS induced decreases in $\alpha_{2A}$-AR mRNA levels after 4 hours. Levels of $\alpha_{2A}$-AR
mRNA were significantly reduced (p<0.05 compared to control) by 100 µM db-cAMP, while 1 mM db-cAMP produced a reduction to about 10% of control values after 4 hours. Levels of α2A-AR mRNA were decreased 60% with 100 µM Sp-cAMPS treatment. The effects of db-cAMP and Sp-cAMPS on steady state α2A-AR mRNA levels were similar to those observed with FSK (Fig. 3-4B), suggesting a common mechanism of action through increases in cyclic AMP levels.

Effects of PMA on α2A-AR mRNA Levels

PKC activation was reported to suppress the intracellular signaling pathway associated with the α2A-AR in platelets (Jakobs et al., 1985; Katada et al., 1985; Watanabe et al., 1985), and to be without effect on α2A-AR regulation in cell lines (Sakaue and Hoffman, 1991; Hamumdzic et al., 1995). Therefore we studied the effects of a PKC activator, PMA, on steady state α2A-AR mRNA levels in astroglial cultures. PMA induced a time-dependent decrease in α2A-AR mRNA levels (Fig. 3-5). The effect of PMA (500 nM) on α2A-AR mRNA levels was noticeable at 1 hour and decreased to about 10% of control at 3 hours. This effect began to wane at 24 hours and levels of α2-AR mRNA approached control values at 48 and 72 hours after treatment. Four hour treatment with 10 nM PMA produced a significant (p<0.05) depression (75% of control) in levels of α2A-AR mRNA, while 50 nM PMA reduced message levels to 10% of control levels (Fig. 3-6). The phorbol ester 4α-phorbol, which does not activate PKC, did not cause any change in α2A-AR mRNA levels (Fig. 3-6A).
Since PMA is known to cause an activation of PKC, we next treated the cells with two different PKC agonists, PDB and Mez. Both PDB and Mez decreased steady state $\text{a}_{\text{2A}}$-AR mRNA levels after a 4 hour treatment (Fig. 3-7). Levels of $\text{a}_{\text{2A}}$-AR mRNA were reduced 80-90% after 50 nM treatment with either drug. PDB and Mez produced reductions in $\text{a}_{\text{2A}}$-AR mRNA levels similar to those observed with PMA (Fig. 3-7B).

In a further set of experiments we tested the combined effects of FSK and PMA on $\text{a}_{\text{2A}}$-AR mRNA levels. Astroglial cultures were treated with concentrations of FSK (100 nM) and PMA (10 nM) which produced approximately half-maximal decreases in $\text{a}_{\text{2A}}$-AR mRNA levels (42% and 45% of control levels, respectively) after 4 hours. When FSK and PMA were applied together, they did not produce significantly greater decreases in levels of $\text{a}_{\text{2A}}$-AR mRNA than the decreases produced when either drug was applied individually (Fig. 3-8).

**Effect of FSK and PMA on Astroglial Morphology**

Treatment of cultured astroglia with FSK or PMA resulted in stellation of the cells (Fig. 3-9). This morphological change was notable within 1 h of treatment and reversed 48-72 h after drug treatment (not shown). FSK treatment produced higher levels of astroglial stellation which lasted longer (Fig. 3-9A) than PMA treatment (Fig. 3-9B). Combined treatment with FSK and PMA produced morphological changes similar to those seen with FSK treatment alone (not shown). These morphological changes were initially thought to contribute to the decreases in levels of $\text{a}_{\text{2A}}$-AR mRNA. However, as will be seen in the next chapter, Epi produced reductions in levels of $\text{a}_{\text{2A}}$-AR mRNA similar to
those obtained with FSK and PMA, but with no accompanying morphological change (Fig. 4-4). Furthermore, the stellation produced by FSK or PMA is reversed 48-72 h after treatment, at which time $\alpha_{2A}$-AR mRNA levels are still significantly reduced (Fig. 3-2 and 3-5). For these reasons, it is unlikely that the observed changes in $\alpha_{2A}$-AR mRNA are due to cell stellation.

**Effect of FSK and PMA on $\alpha_{2A}$-AR mRNA Gene Expression and Stability**

Two possible mechanisms could contribute to the observed decrease in steady state $\alpha_{2A}$-AR mRNA levels: decreased transcription of the gene or decreased stability of the transcript. The effect of FSK and PMA on the rate of transcription was assessed using nuclear runoff assays. The representative blots presented in Fig. 3-10A demonstrate that both FSK and PMA decreased the rate of transcription of $\alpha_{2A}$-AR mRNA by 50% within 1 hour of treatment. Larger (~65%) decreases were detected following 24 hour treatment with FSK or PMA. These results followed the pattern of decrease of $\alpha_{2A}$-AR mRNA steady state levels. This experiment was repeated three times with similar findings. In the results shown in Fig. 3-10, and in the repeat experiments, neither FSK nor PMA treatments significantly changed SOD mRNA transcription.

Stability of $\alpha_{2A}$-AR mRNA was investigated by treating astroglial cultures for 30 min with actinomycin D at a final concentration of 10 mg/ml to inhibit mRNA transcription. The cells were then treated with 10 μM FSK, 500 nM PMA or control solution (0.5% DMSO). Total RNA was extracted hourly for 5 hours and northern blot analysis performed as described in the Chapter 2. The amount of RNA recovered did not
vary over the course of each experiment, nor did the intensity of the SOD signal. The results indicate that FSK decreased α2A-AR mRNA half-life by 36.7 ± 0.1% compared to control values (Fig. 3-12), although the decrease was not statistically significant compared to control values (n=3; p<0.05). PMA, however, significantly (n=3; p < 0.05) increased α2A-AR mRNA half-life by 80.0 ± 0.1% compared with control values. The control half-life was 3.0 ± 0.4 hours (n=6).

Effect of FSK and PMA on Cyclic AMP Accumulation

Experiments with cyclic AMP analogues suggested that increases in intracellular cyclic AMP mediated the effects of FSK. Previous studies also suggested that PKC can directly activate adenylyl cyclase and result in intracellular cyclic AMP accumulation (Kitten et al., 1994; Zhou et al., 1994). Therefore we measured cyclic AMP accumulation in cultured astroglia in response to FSK or PMA treatments.

In the first set of experiments we used a phosphodiesterase inhibitor, IBMX, so that we could detect changes in cAMP synthesis. As expected, FSK caused a time-dependent increase in cyclic AMP accumulation that was significantly (p<0.05) higher than control levels at all times observed (Table 3-1). In contrast, PMA treatment did not significantly increase cyclic AMP levels above control values, suggesting that in cultured astroglia PMA does not effect cAMP synthesis via action at adenylyl cyclase.

Experiments in the absence of IBMX were carried out to determine whether PMA could increase cAMP levels by inhibition of phosphodiesterase activity. PMA treatment did not change cAMP accumulation, but FSK treatment produced time-dependent increases in
cAMP levels (Table 3-2). In order to determine the duration of the FSK-induced increase in cAMP levels we extended FSK treatments to cover the same time-frame used for the mRNA studies. These results showed that cAMP levels peaked 30 min after FSK treatment, and returned to control levels within 1 h (Fig. 3-12). This suggests that the effect of FSK on $\alpha_{2a}$-AR gene expression is rapidly transduced and does not require long-term elevations of cAMP levels to cause long-term decreases in levels of $\alpha_{2a}$-AR mRNA.

**Discussion**

In this study, we have demonstrated that both FSK and PMA treatments decrease steady state levels of $\alpha_{2a}$-AR mRNA in astroglial cultures. This decrease appears to be the result of intracellular cyclic AMP accumulation and PKC activation, respectively. These conclusions are supported by observations that (a) treatment of the cultures with the cyclic AMP analogs, db-cAMP or Sp-cAMPS, elicited decreases in $\alpha_{2a}$-AR mRNA levels similar to those observed with FSK, (b) treatment of the cultures with FSK caused increases in intracellular cyclic AMP concentrations, and (c) treatment of the cultures with the PKC activators, PDB or Mez, caused decreases in $\alpha_{2a}$-AR mRNA levels similar to those seen with PMA. Two possible mechanisms could contribute to these decreases in steady state mRNA levels: decreased transcription of the gene or decreased stability of the transcript. Our studies indicate that both FSK and PMA treatments caused a decrease in the transcription rate of the $\alpha_{2a}$-AR gene.

In HT29 human colonic adenocarcinoma cells, cyclic AMP was shown to transiently increase $\alpha_{2a}$-AR mRNA levels (Sakaue and Hoffman, 1991). In the present
study, our data indicate that increases in cellular cyclic AMP levels induce a decrease in \( \alpha_{2A} \)-AR mRNA levels in astroglia cultured from rat brain. These different results may reflect differences in the lineage and culture conditions of the two cell types. HT29 cells are a human colonic adenocarcinoma cell line, while astroglia used in this study were not transformed and were cultured directly from adult rat brain. It is possible that different regulatory processes operate in transformed cells cultured from the human periphery than in non-tumor cells cultured from the rat CNS. In addition to lineage, the two cell types are maintained in culture differently prior to experimental treatment. For example, HT29 cells were maintained in frequently-changed growth medium containing 7% FBS and were used just after becoming confluent. Astroglia used in the present study were maintained in growth medium containing 10% FBS until four or five days post-confluency, and the growth medium was not changed during the astroglial culture period (13-15 days). The degree of confluence and frequency of growth medium change in HT29 cells may play a role in the regulatory processes seen in these cells. HT29 cells have been shown to increase expression of \( \alpha_{2A} \)-AR mRNA and protein with increasing cell density (Paris et al, 1985; Sakaue and Hoffman, 1994). In addition, expression of \( \alpha_{2A} \)-AR in HT29 cells is reported to decrease with increasing FBS concentration in the growth medium; an effect which was mimicked by insulin and growth factors (Devedjian et al, 1991). Perhaps the cyclic AMP-mediated increase in \( \alpha_{2A} \)-AR mRNA seen by Sakaue and Hoffman (1991) was due to inhibition of the actions of frequent growth medium changes with fresh FBS. In the present study, astroglia were cultured for two weeks without changing the FBS-containing growth medium. Changing the growth media on cultured astroglia resulted in decreases in
\( \alpha_{2A} \)-AR mRNA levels regardless of FBS content (data not shown). Therefore, culture conditions used in the present study may minimize interactions due to frequent growth medium changes.

Using a chloramphenicol acetyl transferase reporter system in JEG-3 cells Sakaue and Hoffman (1991) found that the 5'-flanking region of the human \( \alpha_{2A} \)-AR gene conferred responsiveness to cyclic AMP and PKA. Analysis of the human \( \alpha_{2A} \)-AR promoter region, however, did not reveal a cyclic AMP responsive element (CRE; Handy and Gavras 1992). Similarly, a CRE was suggested but not reported to be present in the promoter region of the rat \( \alpha_{2A} \)-AR gene (Handy et al., 1995). In our studies, FSK treatment caused a decrease in transcription of the \( \alpha_{2A} \)-AR gene as measured by nuclear runoff assay, suggesting the possibility that the gene is regulated differently in cultured astroglia than in HT29 or transfected JEG-3 cells.

Studies have suggested that phorbol esters do not change transcription of the \( \alpha_{2A} \)-AR gene in HT29 cells or pancreatic \( \beta \)-cells (Sakaue and Hoffman, 1991; Hamamdzic et al., 1995). In the present study, PMA decreases transcription and increases the stability of \( \alpha_{2A} \)-AR mRNA in cultured astroglia. Decreased transcription appears to play a greater role, since the net effect of PMA treatment was a decrease in \( \alpha_{2A} \)-AR mRNA steady state levels. In addition, elements which may confer responsiveness to PKC have not been reported for the human or rat \( \alpha_{2A} \)-AR promoter regions, suggesting that PKC activation in astroglia may decrease transcription via some other mechanism.

Previous studies have shown that activated PKC can directly stimulate adenylyl cyclase, resulting in intracellular cyclic AMP accumulation (Kitten et al., 1994; Zhou et
al., 1994). The present study showed that activation of PKC with PMA did not result in intracellular accumulation of cyclic AMP. These results, along with the nearly identical time course of action of PMA and FSK, suggested that the effects of FSK and PMA may converge at some common pathway. We found, however, that the combined effect of PMA and FSK was not strictly additive (Fig. 3-8), arguing against convergence on a common pathway. This raises the possibility that the FSK- and PMA-mediated decreases in rat $\alpha_{2A}$-AR gene transcription observed in the present study and the FSK-mediated increase in transcription of the human $\alpha_{2A}$-AR gene seen by Sakaue and Hoffman (1991) may be due to changes in activity at competing promotor regions. It is possible that PKA and PKC can modulate transcription factors which may compete for the same promotor region of the $\alpha_{2A}$-AR gene. The suppression of $\alpha_{2A}$-AR gene expression would then be dependent on the relative activities of the effected transcription factors.

The human and the rat $\alpha_{2A}$-AR gene contain common motifs in the 2 kb region upstream of the protein coding region. These include a TATA-box, a GC-box, a palindromic region, and several GGAGG repeats (Handy and Gavras, 1992; Handy et al., 1995). The GC-box is a consensus binding site for the nuclear factor Sp1 (Dynan and Tjian, 1983) which acts to enhance transcription. In the rat $\alpha_{2A}$-AR promoter, however, the region overlapping the GC-box was shown to repress gene expression (Handy et al., 1995). This region contains several GGAGG repeats and a related CGAGG sequence (Handy et al., 1995) which have been postulated to bind nuclear factors which can repress gene expression (Giovane et al., 1994; Macleod et al., 1992). Handy et al. (1995) showed that several nuclear factors, including Sp1, bind to the GC-box region. In addition,
phosphorylation of Sp1 by PKC appears to inactivate this transcription factor and lead to decreased DNA binding (Borellini et al., 1990; Leggett et al., 1995). These studies, in light of our results, suggests that cyclic AMP accumulation or PKC activation could result in changes in nuclear factor binding such that transcription of the α2a-AR gene is repressed. Since the effects of cyclic AMP are mediated at the nuclear level by PKA (Riabowol et al., 1988), this kinase may be involved in the changes in transcription we observed.

The long-lasting decrease in levels of α2a-AR mRNA after FSK or PMA treatment is not unusual. Incubation of DDT1 MF-2 hamster vas deferens cells with agents which increase cAMP accumulation leads to a 60% reduction in levels of β2-AR mRNA which persists for at least 72 h (Haddock and Malbon, 1988; Haddock et al., 1989). Similarly, insulin induces a 90% reduction in levels of α2a-AR mRNA which is transcriptionally mediated and lasts 48 h in HT29 cells (Devedjian et al., 1991). In the present study, FSK and PMA also decrease α2a-AR gene expression. In the case of FSK (Fig. 3-12) and Epi (see Chap. 4, Fig. 4-2) these long-term effects on α2a-AR mRNA are mediated by short-term accumulation of cAMP or short-term stimulation of β-AR and α1-AR. These results suggest that the long-term depression of levels of α2a-AR mRNA are mediated by long-term changes in cellular effectors distal to the second messenger pathways. This could include persistent changes in the expression or activity of nuclear trans-acting factors.

One question that arises from the present studies is the identity of endogenous factors which may modulate α2a-AR mRNA via changes in cyclic AMP or PKC activity. Our studies indicate that Epi and angiotensin II (Ang II) treatments cause decreases in
\( \alpha_{2A}\)-AR mRNA levels in cultured astroglia similar to those reported here (Chap. 4). Norepinephrine applied to cultures of astroglia acts at \( \beta \)-AR and \( \alpha_{2A}\)-AR to modulate cyclic AMP formation, and at \( \alpha_1 \)-AR and \( \alpha_{2A}\)-AR to cause phosphoinositide (PI) hydrolysis (Atkinson and Minneman, 1991, 1992; Wilson and Minneman, 1990, 1991). Previous studies from this lab have shown that Ang II also increases PI hydrolysis in cultures of astroglia (Sumners et al., 1991). One of the products of PI hydrolysis is diacylglycerol, which rapidly activates PKC (Nishizuka, 1986). Thus, it appears that endogenous ligands may cause accumulation of cyclic AMP and/or activation of PKC and therefore also regulate \( \alpha_{2A}\)-AR mRNA levels. This hypothesis will be investigated in Chapter 4.

In summary, the present results show that both FSK and PMA treatments decrease \( \alpha_{2A}\)-AR mRNA levels in astroglial cultures. These effects appear to be mediated via two different pathways: increased cyclic AMP concentrations and PKC activation. The implications of these findings for understanding \( \alpha_{2A}\)-AR regulation and the physiological role of \( \alpha_{2A}\)-AR on astroglia will be interesting to explore.
Table 3-1. Effect of FSK and PMA on cyclic AMP accumulation in the presence of IBMX

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (min)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>33.5 ± 5.5</td>
<td>47.5 ± 7.3</td>
<td>67.2 ± 6.8</td>
<td>78.5 ± 8.5</td>
</tr>
<tr>
<td>10 µM FSK</td>
<td>1,460 ± 301*</td>
<td>2,964 ± 576*</td>
<td>5,522 ± 1,014*</td>
<td>6,691 ± 1,038*</td>
</tr>
<tr>
<td>Control</td>
<td>36.7 ± 4.2</td>
<td>42.2 ± 1.7</td>
<td>61.7 ± 4.6</td>
<td>76.5 ± 6.8</td>
</tr>
<tr>
<td>500 nM PMA</td>
<td>45.6 ± 11.0</td>
<td>60.7 ± 8.2</td>
<td>74.6 ± 6.2</td>
<td>89.6 ± 10.6</td>
</tr>
</tbody>
</table>

Values (pmol cyclic AMP/mg protein) are the means ± SE of 4 experiments. Astroglial cultures were incubated at 37°C in DMEM containing 2 mM IBMX with the above for the times indicated. After incubations, cellular cyclic AMP was extracted and analyzed as detailed in Materials and Methods. * significantly different (p < 0.05) from control.

Table 3-2. Effect of FSK and PMA on cyclic AMP accumulation in the absence of IBMX

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (min)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>11.5 ± 0.1</td>
<td>11.1 ± 0.7</td>
<td>10.5 ± 1.4</td>
</tr>
<tr>
<td>10 µM Forskolin</td>
<td>446 ± 119*</td>
<td>947 ± 347*</td>
<td>1,178 ± 475*</td>
</tr>
<tr>
<td>500 nM PMA</td>
<td>11.0 ± 0.9</td>
<td>10.2 ± 0.9</td>
<td>10.9 ± 0.7</td>
</tr>
</tbody>
</table>

Values (pmol cyclic AMP/mg protein) are the means ± SE of 3 experiments. Astroglial cultures were incubated at 37°C in DMEM with the above for the times indicated. After incubations, cellular cyclic AMP was extracted as detailed in Materials and Methods. *significantly different (p < 0.05) from control.
Figure 3-1. Representative northern blot showing relative steady-state levels of the 4.0 kb $\alpha_{2A}$-AR mRNA in 20 $\mu$g total RNA isolated from cultures of astroglia and neurons, and from different brain areas. AG, astroglial cultures; NCult, neuronal cultures; Ctx, cortex; Str, striatum; Hyp, hypothalamus; Cer, cerebellum; BS, brain stem.
Figure 3-2. Effect of FSK on steady-state levels of $\alpha_{2A}$-AR mRNA in cultured astroglia as a function of treatment time. Cultured astroglia were incubated in their growth media in the absence or presence of FSK (10 µM) for the indicated time periods, followed by analysis of $\alpha_{2A}$-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb $\alpha_{2A}$-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (-), control; (+), Epi-treated for the indicated time in h. (B) Quantification of $\alpha_{2A}$-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 3 independent experiments and are presented as % of control levels (100%). Control data did not vary significantly with time and are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 3-3. Effect of FSK on steady-state levels of $\alpha_{2A}$-AR mRNA in cultured astroglia as a function of concentration. Cultured astroglia were incubated in their growth media with vehicle (0.5% DMSO in water) or the indicated concentrations of FSK for 4 hours, followed by analysis of $\alpha_{2A}$-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb $\alpha_{2A}$-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. Dideoxyforskolin (DDF) is included as a negative control. (B) Quantification of $\alpha_{2A}$-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). Control data are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 3-4. Effect of the cyclic AMP analogs Sp-cAMPS and db-cAMP on steady-state levels of α2A-AR mRNA in cultured astroglia. Cultured astroglia were treated with vehicle (water) or the indicated concentrations of Sp-cAMPS or db-cAMP for 4 hours, followed by analysis of α2A-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb α2A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. FSK (250 nM) was included as a positive control. (B) Quantification of α2A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 3-5. Effect of PMA on steady-state levels of α2A-AR mRNA in cultured astroglia as a function of treatment time. Cultured astroglia were incubated in their growth media in the absence or presence of PMA (500 nM) for the indicated time periods, followed by analysis of α2A-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb α2A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (-), control; (+), Epi-treated for the indicated time in h. (B) Quantification of α2A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 3 independent experiments and are presented as % of control levels (100%). Control data did not vary significantly with time and are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 3-6. Effect of PMA on steady-state levels of α2A-AR mRNA in cultured astroglia as a function of concentration. Cultured astroglia were incubated with vehicle (0.5% DMSO in water) or the indicated concentrations of PMA for 4 hours, followed by analysis of α2A-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb α2A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. 4α-phorbol is included as a negative control. (B) Quantification of α2A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). Control data are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 3-7. Effect of the PKC agonists PDB and MEZ on steady-state levels of α2A-AR mRNA in cultured astroglia. Cultured astroglia were treated with vehicle (DMSO in water) or the indicated concentrations of PDB or MEZ for 4 hours, followed by analysis of α2A-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb α2A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. PMA (20 nM) was included as a positive control. (B) Quantification of α2A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 3-8. Effect of combined FSK and PMA treatment on steady-state levels of α2A-AR mRNA in cultured astroglia. Cultured astroglia were treated with vehicle (DMSO in water), 100 nM FSK, 10 nM PMA and a combination of 100 nM FSK and 10 nM PMA for four hours, followed by analysis of α2A-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb α2A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of α2A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 2 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 3-9. Effect of FSK and PMA on morphology of cultured astroglia.

A. Cultured astroglia were incubated with control solution or FSK (10 µM) for 4 or 24 hours at 37°C, followed by preparation of the cells for light microscopy as detailed in Chapter 2. (a) Micrograph of cultured astroglia after 4 h treatment with control solution. (b) Micrograph of cultured astroglia after 4 h treatment with FSK. (c) Micrograph of cultured astroglia after 24 h treatment with FSK. All micrographs are shown enlarged 700X.
Figure 3-9. Continued

B. Cultured astroglia were incubated with control solution or PMA (500 nM) for 4 or 24 hours at 37°C, followed by preparation of the cells for light microscopy as detailed in Chapter 2. (a) Micrograph of cultured astroglia after 4 h treatment with control solution. (b) Micrograph of cultured astroglia after 4 h treatment with PMA. (c) Micrograph of cultured astroglia after 24 h treatment with PMA. All micrographs are shown enlarged 700X.
Figure 3-10. Effect of FSK and PMA on transcription rate of \( \alpha_{2A}\)-AR mRNA.

A. Cultured astroglia were treated with control solution (DMSO in water), 10 \( \mu \)M FSK or 500 nM PMA for the times indicated, followed by nuclear runoff assay as described in Chapter 2. Shown here are representative blots for each treatment at 1, 4, and 24 hours. TE (10 mM tris-HCl pH 8.0/1 mM EDTA) and pGEM7 plasmid were included as negative controls. Similar results were obtained in 3 repeats of these experiments (2 for the 4 h treatment).

- pGEM
- pGEM7 plasmid (1 hour point)
- TE
- (4 and 24 hour points)
- \( \alpha_{2A}\)-AR
- pGEM7 plasmid containing the RG20 (\( \alpha_{2A}\)-AR) cDNA
- SOD
- pUC19 plasmid containing the SOD cDNA
B. Cultured astroglia were treated with control solution (DMSO in water), 10 µM FSK (●) or 500 nM PMA (■) for the times indicated. Quantification of α_{2A}-AR mRNA runoff data normalized against Cu/Zn SOD mRNA runoff data. Data are means ± SE of 3 independent experiments (2 for the 4 h point) and are presented as % of control levels (100%). Control data are plotted on the y-axis.
*significantly different (p<0.05) from control.
Figure 3-11. Effect of FSK and PMA on degradation rate of \( \alpha_{2A} \)-AR mRNA. Cultured astroglia were treated with control solution (DMSO in water, ■), 10 µM FSK (■) or 500 nM PMA (▲) for the times indicated, followed by analysis of \( \alpha_{2A} \)-AR mRNA levels as detailed in Chapter 2. (A) Representative northern blot showing 4.0 kb \( \alpha_{2A} \)-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of \( \alpha_{2A} \)-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means from 3 independent experiments and are presented as % of control levels (100%).
Figure 3-12. Effect of FSK on cAMP levels as a function of time. Cultured astroglia were treated with vehicle control (0.5% DMSO, ◦) or 10 µM FSK (■) for the times indicated. Following this the media were removed, the dishes were washed twice with ice-cold PBS, and cellular cAMP was extracted and analyzed as detailed in Chapter 2. Values are the means ± SE of 4 experiments. * significantly different (p<0.05) compared to control levels.
CHAPTER 4
REGULATION OF $\alpha_{2A}$-ADRENERGIC RECEPTOR mRNA IN CULTURED RAT ASTROGLIA: ROLE OF EPINEPHRINE AND ANGIOTENSIN II

Introduction

Three subtypes of $\alpha_2$-adrenergic receptors ($\alpha_2$-AR), the $\alpha_{2A}$-AR, $\alpha_{2B}$-AR, and $\alpha_{2C}$-AR, have been identified using pharmacological and molecular cloning approaches (Bylund et al., 1994). All three subtypes can be activated by the endogenous catecholamines epinephrine (Epi) and norepinephrine (NE), and inhibit the accumulation of cellular cyclic AMP via inhibition of adenylyl cyclase (Limbird, 1988). The physiological functions of the different subtypes appear to be determined by their pattern of cellular and tissue localizations. For example, activation of $\alpha_{2A}$-AR in the central nervous system appears to mediate decreases in blood pressure (MacMillan et al., 1996), while activation of $\alpha_{2B}$-AR on resistance vessels increases blood pressure (Link et al., 1996). The differences in cell and tissue distribution of $\alpha_2$-AR may also contribute to differing modes of regulation of expression of each subtype. Regulation of central $\alpha_{2A}$-AR represents an important way through which adrenergic signaling is modulated. Up-regulation of $\alpha_{2A}$-AR could potentiate the inhibitory actions of $\alpha_2$-AR agonists, whereas down-regulation may lead to increased activity of adrenergic signaling pathways. An understanding of the regulation of expression of $\alpha_2$-AR in a variety of cell types and
tissues may lead to novel methods to affect changes in the function of $\alpha_{2A}$-AR without changing the expression or function of $\alpha_{2B}$-AR or $\alpha_{2C}$-AR.

Information regarding $\alpha_{2A}$-AR regulation has come largely from the use of cell lines and transfected cells as model systems (Thomas and Hoffman, 1986; Convents et al., 1989; Jones et al., 1990; Sakaue and Hoffman, 1991). Experiments in such transformed cells allow study of receptor regulation within a relatively homogeneous model. However, regulatory processes in transformed cells may not be representative of physiological regulation, and little is known concerning the basic cellular mechanisms of regulation of $\alpha_{2A}$-AR in non-transformed, non-transfected cells. Astroglial cells cultured directly from rat brain are not transformed or transfected and have been shown to contain $\alpha_2$-AR (Richards et al., 1989) which are predominantly of the $\alpha_{2A}$-AR subtype (Chap. 3). In addition, cultured astroglia also contain $\beta$-AR (McCarthy, 1983; Baker et al., 1986) and $\alpha_1$-AR (Hirata et al., 1983; Murphy and Pearce, 1987). These receptors are functionally coupled to signal transduction pathways which include modulation of cyclic AMP accumulation ($\beta$-AR and $\alpha_2$-AR: Baker et al., 1986; Atkinson and Minnemann, 1991, 1992) and increases in phosphoinositide (PI) hydrolysis ($\alpha_1$-AR: Wilson and Minnemann, 1990, 1991). PI hydrolysis leads to increased intracellular calcium and activation of protein kinase C (PKC) via diacylglycerol (Berridge, 1984; Nishizuka, 1986).

Consequently, cultured astroglia provide a good model system for studying the homologous (via $\alpha_2$-AR) or heterologous (via $\alpha_1$-AR or $\beta$-AR) regulation of $\alpha_{2A}$-AR. Using these cells we have recently determined that stimulation of PKC activity with phorbol 12-myristate 13-acetate (PMA) elicits a significant decrease in the steady state
levels of $\alpha_{2A}$-AR mRNA (Chap. 3). Similarly, treatment of cultured astroglia with forskolin (FSK), which increases intracellular cyclic AMP, also causes a decrease in $\alpha_{2A}$-AR mRNA levels (Chap. 3). Both effects are due to reduced transcription of the $\alpha_{2A}$-AR gene. These data suggest that endogenous ligands which stimulate PKC activity or increase intracellular cyclic AMP levels will modulate levels of $\alpha_{2A}$-AR mRNA in cultured astroglia. Thus, in the present study we have investigated the effects of Epi, which stimulates $\alpha_1$, $\alpha_2$, and $\beta$-AR on regulation of $\alpha_{2A}$-AR mRNA levels in cultured astroglia. We have demonstrated that Epi treatment elicits a reduction in $\alpha_{2A}$-AR mRNA, similar to the effects of PMA and FSK. This effect is mediated by combined activation of $\alpha_1$-AR and $\beta$-AR, but not $\alpha_2$-AR. In addition, we showed that angiotensin II (Ang II) treatment of cultured astroglia caused a decrease in levels of $\alpha_{2A}$-AR mRNA. This effect was mediated via Ang II type 1 (AT$_1$) receptor stimulation, which has been shown to result in PKC activation in cultured astroglia (Sumners et al., 1994). Overall, these data suggest that levels of $\alpha_{2A}$-AR mRNA in astroglia undergo heterologous regulation via AT$_1$, $\alpha_1$-AR and $\beta$-AR, and that the intracellular pathways involved include activation of PKC and increases in cyclic AMP.

Results

Effects of Epinephrine on $\alpha_{2A}$-AR mRNA Levels

Epinephrine (Epi) can act via $\alpha_1$-AR, $\alpha_2$-AR, or $\beta$-AR located on the plasma membrane of target cells. It can act via $\beta$-AR to increase cyclic AMP accumulation or via $\alpha_1$-AR to increase PKC activity (Bylund et al., 1994). Considering that increases in
cellular cyclic AMP accumulation or PKC activation led to decreased steady state levels of $\alpha_{2A}$-AR mRNA via decreases in transcription of the $\alpha_{2A}$-AR gene (Chap. 3), we have tested the effects of Epi on $\alpha_{2A}$-AR mRNA levels. Treatment of cultured astroglia with Epi (100 µM) resulted in a time-dependent decrease in steady state levels of $\alpha_{2A}$-AR mRNA (Fig. 4-1). The reduction of $\alpha_{2A}$-AR mRNA levels was significantly different from control levels within 2 h of Epi treatment and reached 10% of control within 4 h. Levels of $\alpha_{2A}$-AR mRNA remained about 10% of control levels through 24 h and then gradually increased toward 50% of control values at 48 and 72 h. Levels of $\alpha_{2A}$-AR mRNA were not significantly different between cultures treated with control vehicle and untreated cultures (data not shown). The Epi-induced decreases in $\alpha_{2A}$-AR mRNA levels were similar in magnitude and timing to the decreases produced by FSK or PMA treatment (Figs. 3-2, 3-5). In order to determine whether the effect of Epi was due to prolonged exposure of cultured astroglia to this catecholamine, we conducted a set of pulse-chase experiments. Cultured astroglia were treated with Epi (100 µM) for 10 min, at which time the media was removed and replaced with untreated (Epi-free) conditioned media. The resulting decreases in levels of $\alpha_{2A}$-AR mRNA were similar in magnitude and timing of onset as those seen with prolonged Epi treatment (Fig. 4-2). Recovery of $\alpha_{2A}$-AR mRNA levels, however occurred more rapidly, reaching control levels 72 h after Epi treatment. The Epi-induced decrease in $\alpha_{2A}$-AR mRNA levels was also concentration-dependent, coinciding with increases in cellular cyclic AMP (Fig. 4-3), a second messenger associated with $\beta$-AR stimulation. Levels of $\alpha_{2A}$-AR mRNA were significantly lower than control with 10 nM Epi treatment, while 100 nM Epi elicited a maximal effect and reduced the
levels to about 10% of control values. The adrenergic receptor agonist NE elicited similar concentration-dependent decreases in levels of $\alpha_{2A}$-AR mRNA (Fig. 4-3B).

**Effect of Epi on Astroglial Morphology**

Treatment of cultured astroglia with FSK or PMA resulted in stellation of the cells (Fig. 3-9). This morphological change was thought to contribute to the decreases in steady-state levels of $\alpha_{2A}$-AR mRNA. Similar decreases in the levels of $\alpha_{2A}$-AR mRNA were seen with Epi treatment, but without the accompanying stellation (Fig. 4-4). Similarly, Ang II treatment caused decreases in the levels of $\alpha_{2A}$-AR mRNA (Fig. 4-12), but did not change the morphology of the cultured astroglia (not shown). Once again, these data suggest that the observed changes in levels of $\alpha_{2A}$-AR mRNA are not due to cell stellation.

**Effect of Pertussis Toxin (PTX) on the Epi-Induced Decrease of $\alpha_{2A}$-AR mRNA Levels**

It is well known that $\alpha_2$-AR inhibit adenylyl cyclase via $G_i$. In order to determine whether the Epi-induced decrease of levels of $\alpha_{2A}$-AR mRNA was mediated through $G_i$-coupled pathways we pretreated cultured astroglia with PTX (200 ng/ml, 24 h), which inactivates both $G_i$ and $G_o$ (Milligan, 1988). Treatment of cultured astroglia with PTX did not reverse the Epi-induced decrease in levels of $\alpha_{2A}$-AR mRNA (Fig. 4-5), suggesting that Epi did not act solely via a $G_i$-coupled receptor. PTX treatment alone did not alter levels of $\alpha_{2A}$-AR mRNA.
Effect of Adrenergic Receptor Antagonists on the Epi-induced Decrease of $\alpha_{2A}$-AR mRNA Levels

Because Epi can act via $\beta$-AR, $\alpha_2$-AR or $\alpha_1$-AR we assessed the effects of various adrenergic receptor antagonists on the Epi-induced decreases in $\alpha_{2A}$-AR mRNA levels. Previous results suggested that activation of $\beta$-AR or $\alpha_1$-AR may lead to decreased expression of $\alpha_{2A}$-AR mRNA (Chap. 3). In addition, treatment of cultured astroglia with the $\beta$-AR agonist isoproterenol produced decreases in levels of $\alpha_{2A}$-AR mRNA (data not shown). However, treatment of cultured astroglia with the $\beta$-AR antagonist propranolol (Prop; Fig. 4-6) or the $\alpha_1$-AR antagonist prazosin (Praz; Fig. 4-7) alone did not inhibit the effect of Epi, suggesting that Epi did not exert its effect via either receptor exclusively. In addition, this effect of Epi was not inhibited by the $\alpha_2$-AR antagonists rauwolscine (not shown) or yohimbine (Yoh; Fig. 4-8). Because earlier studies had implicated signal transduction pathways associated with $\alpha_1$-AR and $\beta$-AR stimulation (Chap. 3), we suspected that the Epi-induced effect was mediated via these two receptors. Therefore, we treated cultured astroglia with a combination of Prop and Praz, and found that together they completely suppressed the Epi-induced decrease in levels of $\alpha_{2A}$-AR mRNA (Fig. 4-9). In a further set of experiments we treated cultured astroglia with Epi plus the combined $\alpha_1$-AR and $\beta$-AR antagonist labetalol. Labetalol also reversed the Epi-induced decrease in levels of $\alpha_{2A}$-AR mRNA (Fig. 4-10) providing additional evidence that Epi exerted its effect via both $\alpha_1$-AR and $\beta$-AR. To further delineate the involvement of the signaling pathways associated with $\alpha_1$-AR and $\beta$-AR in the Epi-induced effect we treated
cultured astroglia with the PKA inhibitor H-89 in combination with Praz. Praz and H-89 treatment reversed the Epi-induced decrease in levels of $\alpha_{2A}$-AR mRNA (Fig. 4-11), providing further evidence for the involvement of signaling pathways associated with $\alpha_1$-AR and $\beta$-AR.

**Effect of Angiotensin II on $\alpha_{2A}$-AR mRNA Levels**

Angiotensin II (Ang II) has been reported to have a negative regulatory effect on $\alpha_2$-AR in the nucleus tractus solitarius (Fior et al., 1994), a region which expresses mRNA for $\alpha_{2A}$-AR and $\alpha_{2C}$-AR (Nicholas et al., 1993; Scheinin et al., 1994). This effect of Ang II was mediated via the Ang II type 1 (AT$_1$) receptor, a receptor which is coupled to PI hydrolysis and the subsequent activation of PKC (Sumners et al., 1996). Ang II can also act via AT$_1$ receptors in cultured astroglia to stimulate PKC activity (Sumners et al., 1994). Because we had previously shown that activation of PKC decreased levels of $\alpha_{2A}$-AR mRNA in cultured astroglia (Chap. 3), we investigated the role of Ang II in regulating levels of $\alpha_{2A}$-AR mRNA.

Treatment of cultured astroglia with Ang II for 4 h decreased steady state levels of $\alpha_{2A}$-AR mRNA (Fig. 4-12). This effect was blocked by the AT$_1$ receptor antagonist losartan and not by the Ang II type 2 receptor antagonist PD 123319 (Fig. 4-13). These results indicated that Ang II exerted its effect via the AT$_1$ receptor, suggesting involvement of PKC in the decrease of $\alpha_{2A}$-AR mRNA levels.
Discussion

The goal of this study was to investigate the regulation of steady-state levels of $\alpha_{2A}$-AR mRNA by ligands which act via cell-surface receptors to increase cellular cyclic AMP accumulation or PKC activation. These investigations were prompted by earlier studies which showed that increases in cellular cyclic AMP accumulation or PKC activation decreased steady-state levels of $\alpha_{2A}$-AR mRNA (Chap. 3). The agents we tested were Epi and Ang II. Epi can act via $\alpha_1$-AR to stimulate PI hydrolysis and subsequently, PKC activation, or via $\alpha_2$-AR and $\beta$-AR to decrease or increase cAMP accumulation, respectively (Bylund et al., 1994). Ang II can act via $AT_1$ receptors to stimulate PI hydrolysis and subsequently, PKC activation (Sumners et al., 1996).

In the present study, we have shown that Epi and Ang II treatment of cultured astroglia elicits a decrease in the levels of $\alpha_{2A}$-AR mRNA similar to the decrease caused by FSK or PMA treatment. The Epi-induced decrease was mediated by activation of $\beta$-AR and $\alpha_1$-AR, while Ang II treatment decreases levels of $\alpha_{2A}$-AR mRNA via $AT_1$ receptor stimulation.

Experiments in HT29 cells and cultured astroglia indicate that steady state levels of $\alpha_{2A}$-AR mRNA are regulated in a heterologous fashion at the level of gene transcription. Cyclic AMP causes an increase in transcription of the $\alpha_{2A}$-AR gene in HT29 cells (Sakaue and Hoffman, 1991) and a decrease in cultured astroglia (Chap. 3). In addition, PKC activation decreases transcription in cultured astroglia (Chap. 3) but is without effect in HT29 cells (Sakaue and Hoffman, 1991). Changes in degradation rate of the $\alpha_{2A}$-AR
transcript do not appear to play a major role in steady state levels of $\alpha_{2A}$-AR mRNA in either cell type. The opposite effect of cyclic AMP on the regulation of $\alpha_{2A}$-AR mRNA observed in cultured astroglia compared to transformed cells may represent cell-, tissue-, or species-specific regulation of this gene.

The regulatory effects of cAMP and PKC in cultured astroglia were also implied after treatment with Epi. Concentration-dependent increases in cellular cAMP accompanied Epi treatment of cultured astroglia, suggesting stimulation of $\beta$-AR. However, the Epi-induced decrease in levels of $\alpha_{2A}$-AR mRNA could not be blocked with the $\beta$-AR antagonist propranolol alone. Similarly, $\alpha_1$-AR and $\alpha_{2A}$-AR antagonists alone were not able to block the Epi-induced decrease in levels $\alpha_{2A}$-AR mRNA. Further evidence that $\alpha_{2A}$-AR were not involved was provided by the inability of PTX to suppress the Epi-induced effect.

We also treated cultured astroglia with a combination of Praz and Prop because activation of signal transduction pathways associated with $\alpha_1$-AR and $\beta$-AR produce decreases in levels of mRNA similar to those seen with Epi treatment. Complete reversal of the Epi-induced effect was seen with combined Praz and Prop treatment. Similarly, the action of Epi was inhibited by treatment of cultured astroglia with other agents which specifically antagonize $\alpha_1$-AR and $\beta$-AR or their intracellular pathways. Combined with the results of our previous study showing that cAMP and PKC decrease levels of $\alpha_{2A}$-AR mRNA (Chap. 3), the present results suggest that Epi can lead to decreased transcription on the $\alpha_{2A}$-AR gene via activation of $\alpha_1$-AR and $\beta$-AR and their intracellular signaling.
pathways. These results also suggest that levels of $\alpha_{2A}$-AR mRNA are not regulated in a homologous fashion in cultured astroglia by stimulation of $\alpha_{2A}$-AR.
Figure 4-1. Effect of Epi on steady-state levels of α2A-AR mRNA in cultured astroglia as a function of treatment time. Cultured astroglia were incubated with control solution (100 µM ascorbate in PBS) or Epi (100 µM) for the indicated time periods at 37°C, followed by analysis of α2A-AR mRNA levels as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb α2A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (-), control; (+), Epi-treated for the indicated time in h. (B) Quantification of α2A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). Control data are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 4-2. Pulse-Chase effect of Epi on steady-state levels of $\alpha_{2A}$-AR mRNA in cultured astroglia as a function of treatment time. Cultured astroglia were incubated with control solution or Epi (100 µM) for 10 min at 37°C. Following this the media were removed and replaced with conditioned (Epi-free) media for the indicated time periods, followed by analysis of $\alpha_{2A}$-AR mRNA levels as detailed in Materials and Methods. (A) Representative Northern blot showing 4.0 kb $\alpha_{2A}$-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (-), control; (+), Epi-treated for the indicated time in h. Cultured astroglia which did not have the media removed and replaced are labeled untouched (UT). (B) Quantification of $\alpha_{2A}$-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). Control data are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 4-3. Effect of Epi or NE on steady-state levels of $\alpha_{2A}$-AR mRNA in cultured astroglia as a function of concentration. Cultured astroglia were incubated with control solution or the indicated concentrations of Epi (○) or NE (♦) for 4 h at 37°C, followed by analysis of $\alpha_{2A}$-AR mRNA levels as detailed in Materials and Methods. (A) Representative Northern blot showing 4.0 kb $\alpha_{2A}$-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation with Epi. (B) Quantification of $\alpha_{2A}$-AR mRNA data normalized against Cu/Zn SOD mRNA. Also included the levels of cellular cyclic AMP measured as a function of Epi concentration (□). Data are means ± SE from 4 independent experiments in each case (1 experiment for NE) and are presented as % of control levels (100%) for mRNA data and as pmol/mg protein for cyclic AMP data. Control data are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 4-4. Effect of Epi on morphology of cultured astroglia. Cultured astroglia were incubated with control solution or Epi (100 µM) for 4 or 24 hours at 37°C, followed by preparation of the cells for light microscopy as detailed in Chapter 2. (A) Micrograph of cultured astroglia after 4 h treatment with control solution. (B) Micrograph of cultured astroglia after 4 h treatment with Epi. (C) Micrograph of cultured astroglia after 24 h treatment with Epi. All micrographs are shown enlarged 700X.
Figure 4-5. Effect of pertussis toxin on Epi-induced decrease of \( \alpha_{2A} \)-AR mRNA levels. Cultured astroglia were treated with control solution (water) or with 200 ng/ml PTX for 24 h at 37°C. Cells were then treated with control solution (100 µM ascorbate in PBS) or Epi (10 nM) for 4 hours. Analysis of \( \alpha_{2A} \)-AR mRNA levels was then completed as detailed in Chapter 2. Shown is quantification of \( \alpha_{2A} \)-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are from 1 experiment and are presented as % of control levels (100%).
Figure 4-6. Effect of the β-AR antagonist propranolol on Epi-induced decrease of α_{2A}-AR mRNA levels. Cultured astroglia were treated for 15 min with the indicated concentrations of propranolol (Prop) followed by 10 nM Epi treatment for 4 hours. Analysis of α_{2A}-AR mRNA levels was then completed as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb α_{2A}-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of α_{2A}-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 4-7. Effect of the α₁-AR antagonist prazosin on Epi-induced decrease of α₂₆-AR mRNA levels. Cultured astroglia were treated for 15 min with the indicated concentrations of prazosin (Praz) followed by 10 nM Epi treatment for 4 hours. Analysis of α₂₆-AR mRNA levels was then completed as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb α₂₆-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of α₂₆-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 4-8. Effect of the α₂-AR antagonist yohimbine on Epi-induced decrease of α₂A-AR mRNA levels. Cultured astroglia were treated for 15 min with the indicated concentrations of yohimbine (Yoh) followed by 10 nM Epi treatment for 4 hours. Analysis of α₂A-AR mRNA levels was then completed as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb α₂A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of α₂A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 4-9. Epi-induced decrease of $\alpha_{2A}$-AR mRNA levels: Effect of combined propranolol and prazosin treatment. Cultured astroglia were treated with control solution (0.01% methanol in water), or with a combination of Prop and Praz (1 $\mu$M each) for 15 min at 37°C. Cells were then treated with control solution (100 $\mu$M ascorbate in PBS) or Epi (10 nM) for 4 h. This was followed by analysis of $\alpha_{2A}$-AR mRNA levels as detailed in Materials and Methods. (A) Representative Northern blot showing 4.0 kb $\alpha_{2A}$-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of $\alpha_{2A}$-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 3 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 4-10. Effect of the β-AR and α₁-AR antagonist labetalol on Epi-induced decrease of α₂A-AR mRNA levels. Cultured astroglia were treated with control solution or labetalol (1 µM) for 15 min at 37°C. Cells were then treated with control solution (100 µM ascorbate in PBS) or Epi (10 nM) for 4 hours. Analysis of α₂A-AR mRNA levels was then completed as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb α₂A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of α₂A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
Figure 4-11. Effect of the protein kinase A inhibitor H-89 and prazosin on Epi-induced decrease of α_{2A}-AR mRNA levels. Cultured astroglia were treated with control solution (0.01% methanol) or with H-89 (250 nM) and prazosin (1 µM) for 15 min at 37°C. Cells were then treated with control solution (100 µM ascorbate in PBS) or with Epi (10 nM) for 4 hours. Analysis of α_{2A}-AR mRNA levels was then completed as detailed in Chapter 2. Shown is quantification of α_{2A}-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are from 1 experiment and are presented as % of control levels (100%).
Figure 4-12. Effect of Ang II on steady-state levels of α₂A-AR mRNA in cultured astroglia as a function of concentration. Cultured astroglia were incubated with control solution (water) or the indicated concentrations of Ang II for 4 hours, followed by analysis of α₂A-AR mRNA levels as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb α₂A-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of α₂A-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). Control data are plotted on the y-axis. *significantly different (p < 0.05) from control.
Figure 4-13. Effect of Losartan and PD 123319 on Ang II-induced decrease of \( \alpha_{2A}\)-AR mRNA levels. Cultured astroglia were treated with control solution (water) or with Losartan or PD 123319 (1 \( \mu \)M each) for 15 min at 37°C. Cells were then treated with control solution (water) or Ang II (100 nM) for 4 hours. Analysis of \( \alpha_{2A}\)-AR mRNA levels was completed as detailed in Chapter 2. (A) Representative Northern blot showing 4.0 kb \( \alpha_{2A}\)-AR (RG20) mRNA and 0.7 kb Cu/Zn SOD mRNA in each treatment situation. (B) Quantification of \( \alpha_{2A}\)-AR mRNA data normalized against Cu/Zn SOD mRNA. Data are means ± SE from 4 independent experiments and are presented as % of control levels (100%). *significantly different (p < 0.05) from control.
CHAPTER 5
REGULATION OF $\alpha_{2A}$-ADRENERGIC RECEPTOR NUMBER BY EPINEPHRINE

Introduction

Studies have shown that a variety of cellular mechanisms can lead to changes in the number or sensitivity of receptors on the cell surface. These changes can involve uncoupling of the receptor from its G protein, sequestration of the receptor away from the cell surface, degradation of existing receptors, and reduced synthesis of new receptors.

The most rapid way of uncoupling a G protein-coupled receptor from its G protein is via agonist-dependent phosphorylation of the receptor by G protein coupled receptor kinases. The most studied members of this kinase family are the $\beta$-adrenergic receptor kinases ($\beta$ARK), which have been shown to phosphorylate $\beta_2$-AR (Benovic et al., 1986). $\beta$ARK phosphorylates only the agonist occupied $\beta_2$-AR (Benovic et al., 1986) leading to enhanced affinity of the receptor for $\beta$-arrestin (Lohse et al., 1990). Binding of $\beta$-arrestin inhibits the receptor from interacting with its stimulatory G protein, effectively uncoupling the receptor from its signal transduction pathway (Lohse et al., 1992). This type of homologous regulation by $\beta$ARK has also been described for G$_i$-coupled receptors such as the D$_2$-dopamine receptor (Bates et al., 1991), the m2-muscarinic receptor (Richardson and Hosey, 1992), the 5-HT$_{1B}$-serotonin receptor (Pleus and Bylund, 1992), and the $\alpha_{2A}$-AR (Liggett et al., 1992).
In addition to βARK-mediated homologous desensitization, G protein-coupled receptors can be subject to heterologous desensitization via effector kinases such as PKA or PKC. Phosphorylation of the β_2-AR by PKA (Benovic et al., 1985, 1987; Bouvier et al., 1987, 1989) or PKC (Pitcher et al., 1992; Bouvier et al., 1991) are not dependent on agonist occupancy of the receptor. Activation of these kinases by any pathway can lead to β_2-AR phosphorylation (Benovic et al., 1985). These mechanisms appear to occur more slowly (minutes to hours) than homologous desensitization (seconds to minutes). PKA and PKC preferentially phosphorylate receptor sites in the third intracellular loop thought to be responsible for interaction with the G protein (Pitcher et al., 1992; Okamoto et al., 1991; Bouvier et al., 1987; Johnson et al., 1990). Therefore, these kinases appear to desensitize the receptor via uncoupling it from its G protein.

Also occurring over a time-frame of minutes to hours is the agonist-induced translocation of β_2-AR to intracellular compartment (Perkins et al., 1991). The molecular mechanisms for this sequestration are largely unknown, but appear to involve site in the cytoplasmic tail of the β_2-AR (Hausdorff et al., 1990). Such sequestration also occurs for the α_2A-AR (Liggett et al., 1992; Eason and Liggett, 1992).

Long-term agonist exposure leads not only to sequestration of G protein-coupled receptors, but also to downregulation of receptor number. Similarly, activation of PKA or PKC can lead to changes in the amount of a receptor at the cell surface. These processes are slow (hours to days) and can involve increased receptor degradation and decreased receptor synthesis. Long-term agonist exposure can elicit homologous downregulation of β_2-AR via increased degradation of the receptor (Bouvier et al., 1985). Similarly, α_2A-AR
undergo agonist-induced downregulation (Eason and Liggett, 1992). Degradation of β2-AR can also be enhanced by PKA or PKC-mediated phosphorylation (Bouvier et al., 1985; Pitcher et al., 1992; Johnson et al., 1990).

Downregulation of receptor number can also occur as a result of decreased steady-state levels of receptor mRNA and subsequent decreased de novo synthesis of receptors. Decreased gene transcription and increased mRNA degradation can contribute to decreases in levels of mRNA. For example, reduction of β2-AR mRNA leads to downregulation of the number of β2-AR (Hadcock and Malbon, 1988; Bouvier et al., 1989). This reduction in mRNA levels is mediated by decreased stability of the transcript and not by decreased transcription rate (Hadcock et al., 1989). These decreases in levels of mRNA are the result of increased levels of cellular cAMP (Hadcock and Malbon, 1988; Hadcock et al., 1989; Bouvier et al., 1989), implying activation of PKA because this kinase mediates the intracellular effects of cAMP (Riabolwol et al., 1988).

We have shown similar heterologous downregulation of steady-state levels of α2A-AR mRNA in cultured astroglia. Accumulation of cAMP and activation of PKC lead to decreased levels of α2A-AR mRNA via decreased transcription and not via increased degradation of the transcript (Chap. 3). The effect on mRNA levels could be mimicked by Epi treatment of the cultured astroglia. Epi elicited these changes via α1-AR and β-AR providing further evidence for the heterologous nature of this regulation. Therefore, we investigated whether treatment of cultured astroglia with Epi, or with agents that cause activation of PKC or accumulation of cAMP lead to decreases in the number of α2A-AR.
We found that the $\alpha_{2A}$-AR number is subject to heterologous regulation, and that the effect of Epi on $\alpha_{2A}$-AR number may have a homologous component.

**Results**

**Effects of FSK, PMA and Epi on $\alpha_2$-AR Specific Binding in Cultured Astroglia**

In the present study we investigated whether Epi and agents that increased PKC activity or intracellular cyclic AMP levels cause a decrease in $\alpha_2$-AR specific binding, consistent with the observed effects on $\alpha_{2A}$-AR mRNA. Binding of $\alpha_2$-AR was analyzed using $[^3H]$-MK 912 as the radioactive ligand. Binding of $[^3H]$-MK 912 to membranes prepared from untreated cultured astroglia was linear with respect to protein concentrations from 2-25 µg. At 25°C the binding of $[^3H]$-MK 912 reached equilibrium within 30 min and remained stable for at least 2 h. Thus, in subsequent experiments, 10 µg of protein were used during an incubation time of 1 h.

Incubation of cultured astroglia with FSK (10 µM; 2-72 h) or PMA (500 nM; 2-72 h), agents that increase intracellular cyclic AMP levels or increase PKC activity respectively, elicited a time-dependent decrease in the level of specific $[^3H]$-MK 912 binding (Fig. 5-1). Similar changes in binding were obtained when using either 0.2 nM $[^3H]$-MK 912 (Fig. 5-1) or 5 nM $[^3H]$-MK 912 (data not shown). For example, binding of $[^3H]$-MK 912 was significantly reduced (p < 0.05 compared to control) 8 h after PMA treatment and 16 h after FSK treatment, and reached approximately 60% of control levels 24 h after FSK or PMA treatment. At 48 and 72 h after PMA treatment $[^3H]$-MK 912 binding had returned to approximately 80% of control levels, while membranes from FSK-
treated astroglia exhibited a further decrease in specific binding. In order to determine saturation binding parameters, membranes prepared from cultured astroglia treated with control solution (0.5% DMSO), FSK (10 µM) or PMA (500 nM) for 24 h were incubated with increasing concentrations of [³H]-MK 912 (0.1-10 nM) at 25°C for 1 h. Figure 5-2A depicts a representative experiment showing that specific binding of [³H]-MK 912 increased with increasing concentration of radiolabeled ligand and was saturable in each case. The data also indicate that the level of binding was less in FSK- or PMA-treated cells compared with controls. Scatchard analysis of this data (Fig. 5-2B) revealed that the $B_{\text{max}}$ values for [³H]-MK 912 binding were 2.92, 1.65, and 1.23 pmol/mg protein for the control, FSK, and PMA-treated groups, respectively. The $K_D$-values did not change among the control, FSK, or PMA-treated groups (0.55, 0.58 and 0.53 nM, respectively). This experiment was repeated 4 times with similar results for the $K_D$ and $B_{\text{max}}$ values (Table 5-1), indicating that FSK and PMA pretreatment significantly ($p<0.05$ compared to control) decreased the number of $\alpha_{2A}$-AR without changing their affinity.

Similar saturation binding assays were conducted with membranes from cultured astroglia treated with either control solution (100 µM ascorbate in PBS) or Epi (10 µM) for 24 h. Figure 5-3A shows a representative saturation experiment which demonstrates that [³H]-MK 912 specific binding increased with increasing concentrations of radiolabeled ligand and was saturable in both control and Epi-treated cells. Scatchard analysis of the saturation binding data revealed that Epi treatment produced nearly an 85% decrease in the number of [³H]-MK 912 binding sites (compared to control) without effecting the affinity of those sites (Fig. 5-3B; Table 5-1). The data presented in Table 5-1 also indicate
that Epi-treatment produced larger decreases (~85%) in $B_{\text{max}}$ values than FSK (~35%) or PMA (~55%) treatment, suggesting that activation of both pathways may be necessary to maximally reduce [$^{3}$H]-MK 912 binding.

In order to determine if activation of both pathways reduced [$^{3}$H]-MK 912 binding to the same extent as Epi, saturation binding assays were conducted with membranes from cultured astroglia treated with either control solution (1% DMSO) or a combination of FSK (10 $\mu$M) and PMA (500 nM). The results of a representative saturation experiment are shown in Figure 5-4A, and demonstrate that specific binding of [$^{3}$H]-MK 912 increased with increasing concentration of radiolabeled ligand and was saturable in both cases. The data also indicate that the level of binding was less in the cells treated with FSK and PMA than in the controls. Scatchard analysis of the saturation binding data (Fig. 5-4B) revealed that the $B_{\text{max}}$ values for [$^{3}$H]-MK 912 binding were 2.70 and 1.44 pmol/mg protein for the control and FSK/PMA-treated groups respectively. The $B_{\text{max}}$ value for the FSK/PMA-treated cells was similar to that seen in cells treated with either drug individually. The $K_{D}$ values did not vary between the control and FSK/PMA-treated groups (0.81 and 0.80 nM, respectively). This experiment was repeated twice with similar results for the $K_{D}$ and $B_{\text{max}}$ values (Table 5-1). These results suggest that increases in intracellular cyclic AMP and PKC activation are not sufficient to account for all of the decreases in [$^{3}$H]-MK 912 binding observed with Epi treatment.
TABLE 5-1. Summary of the $K_p$ and $B_{\text{max}}$ values obtained for $[^3\text{H}]-\text{MK 912}$ specific binding to cultured astroglia following FSK, PMA, and Epi treatments

<table>
<thead>
<tr>
<th></th>
<th>$K_p$ (nM)</th>
<th>$B_{\text{max}}$ (fmol/mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.69 ± 0.11</td>
<td>2.73 ± 0.20</td>
</tr>
<tr>
<td>Forskolin (10 µM; 24 h)</td>
<td>0.86 ± 0.23</td>
<td>1.75 ± 0.24*</td>
</tr>
<tr>
<td>PMA (500 nM; 24 h)</td>
<td>0.57 ± 0.04</td>
<td>1.17 ± 0.11*</td>
</tr>
<tr>
<td>Control</td>
<td>0.51 ± 0.01</td>
<td>2.96 ± 0.27</td>
</tr>
<tr>
<td>Epinephrine (10 µM; 24 h)</td>
<td>0.42 ± 0.10</td>
<td>0.50 ± 0.07*</td>
</tr>
</tbody>
</table>

$[^3\text{H}]-\text{MK 912}$ specific binding was determined in control-, FSK-, PMA-, and Epi- treated cultured astroglia as detailed in Materials and Methods. Data are mean ± SEM values of 4 experiments. * Significantly different from controls (p < 0.05)

Effect of Downregulating $\alpha_{2A}$-AR on cAMP Accumulation

The $\alpha_{2}$-AR are coupled to multiple signaling pathways (Enkvist et al., 1996). For example, activation of $\alpha_{2}$-AR by NE or Epi can lead to activation of potassium channels (Aghajanian and VanDerMaelen, 1982), inhibition of calcium channels (Holz et al., 1986), mobilization of intracellular calcium stores (Michel et al., 1989), and stimulation of PI hydrolysis (Wilson and Minneman, 1991; Cotecchia et al., 1990). In most cells, activation of $\alpha_{2}$-AR has been shown to cause a reduction in cellular cAMP levels as a result of inhibition of adenylyl cyclase (Limbird, 1988). Considering this, we tested whether the $\alpha_{2A}$-AR-mediated decrease in cAMP levels in cultured astroglia was modified in cells where the levels of $\alpha_{2A}$-AR had been downregulated. In the first series of experiment, we tested whether activation of $\alpha_{2A}$-AR caused a decrease in cAMP levels in cultured astroglia. Incubation of cultured astroglia with the $\alpha_{2}$-AR agonist clonidine (1 nM - 10
µM; 20 min) resulted in no significant changes in basal levels of cellular cAMP (data not shown). By contrast, in cultures that were treated with FSK (10 µM) for 20 min to raise intracellular cAMP levels, clonidine (1 nM - 10 µM) caused a decrease in those levels of cAMP (Fig. 5-5). In the next series of experiments, we tested whether pretreatment of cultured astroglia with 10 µM Epi for 24 h, to decrease the number of α₂A-AR present, would reduce the inhibitory effects of clonidine on cellular cAMP levels. The data presented in Figure 5-5 also show that incubation of Epi-pretreated cells with clonidine (1 nM - 10 µM; 20 min) caused a decrease in FSK-stimulated cAMP levels. However, this decrease was not significantly different than the decrease obtained with clonidine in cells not pretreated with Epi (Fig. 5-5). These data suggest that the reduction in α₂A-AR produced by Epi pretreatment does not include α₂A-AR that are coupled to a fall in cellular cAMP levels.

Discussion

Downregulation of G-protein coupled receptors can occur as a result of decreased receptor synthesis or increased receptor degradation (Bouvier et al., 1989; Collins et al., 1990; Lohse, 1993). These processes can be initiated by agonist binding to the receptor (homologous regulation) or by activation of intracellular signaling pathways (heterologous regulation). Previous studies (see chaps. 3 and 4) in cultured astroglia showed that levels of α₂A-AR mRNA were downregulated in a heterologous fashion via activation of α₁-AR or β-AR, or via activation of their intracellular signaling pathways with PMA or FSK. The decreased expression of α₂A-AR mRNA in response to PMA, FSK or Epi treatment
suggested that these agents may lead to decreased \textit{de novo} synthesis of $\alpha_{2A}$-AR. The results presented here demonstrate that FSK, PMA and Epi all elicit decreases in the number of $\alpha_{2A}$-AR, but those decreases differ depending on the agent. In addition, downregulation of $\alpha_{2A}$-AR number with Epi led to changes in the ability of an $\alpha_{2A}$-AR agonist to inhibit cAMP accumulation.

Treatment of cultured astroglia with FSK, PMA or Epi induced similar decreases in the levels of $\alpha_{2A}$-AR mRNA. Changes in cell surface receptor expression can be the combined result of changes in \textit{de novo} synthesis of receptors and changes in degradation of existing receptors. Given that Epi, FSK and PMA produce similar heterologous decreases in levels of $\alpha_{2A}$-AR mRNA, and that steady state levels of mRNA influence \textit{de novo} synthesis rates, it is possible that all three agents decrease \textit{de novo} synthesis to an equal extent. Minimal levels of $\alpha_{2A}$-AR mRNA may reduce \textit{de novo} synthesis of receptors to minimal levels allowing for disappearance of existing receptors in accordance with receptor half-life. We found that 24 h FSK or PMA treatment reduced $\alpha_{2A}$-AR number approximately 40-50% suggesting that the half-life of $\alpha_{2A}$-AR in cultured astroglia may be similar to that observed (26 h) in HT29 cells (Paris et al., 1987). Epi treatment, however, reduced $\alpha_{2A}$-AR number to a greater extent than FSK or PMA treatment. We initially thought that the difference was due to activation of both the cyclic AMP and PKC signaling pathways by Epi. However, activation of both of these pathways by combined FSK/PMA treatment did not decrease $\alpha_{2A}$-AR number more than either treatment alone (Fig. 5-4, Table 5-1). This suggests that Epi, FSK and PMA may mediate different changes in the degradation of existing $\alpha_{2A}$-AR.
Phosphorylation of the $\beta_2$-AR by cyclic AMP dependent protein kinase (PKA) and PKC has been shown to play a role in $\beta_2$-AR downregulation (Bouvier et al., 1987, 1989). However, consensus sequences for PKA or PKC-mediated phosphorylation have not been reported for either the human (Eason and Liggett, 1992) or the rat $\alpha_2A$-AR (Lanier et al., 1991). $\beta$ARK plays a role in homologous desensitization of $\beta_2$-AR (Benovic et al., 1986) and the human $\alpha_2A$-AR (Benovic et al., 1987). The amino acid sequence LEESSSS in the third cytoplasmic loop of the human $\alpha_2A$-AR has been identified as the substrate for $\beta$ARK phosphorylation (Oronato et al., 1991). Removal of this site eliminates agonist-dependent phosphorylation and desensitization of the $\alpha_2A$-AR (Liggett et al., 1992). The rat $\alpha_2A$-AR contains the same sequence in the third cytoplasmic loop (Lanier et al., 1991), suggesting that the rat $\alpha_2A$-AR is subject to homologous regulation. Such homologous regulation could explain the differences we observed in $\alpha_2A$-AR number in the Epi-treated cells compared to the FSK- or PMA-treated cells. Epi treatment of cultured astroglia could result in combined heterologous decreases in $\alpha_2A$-AR synthesis via $\alpha_1$-AR and $\beta$-AR and their signaling pathways, and homologous increases in $\alpha_2A$-AR phosphorylation, sequestration, and degradation. Additional studies are needed to further delineate the effect of these two modes of regulation on $\alpha_2A$-AR expression.

Because Epi induced large decreases in $\alpha_2A$-AR number, we hypothesized that $\alpha_2A$-AR responsiveness would be decreased after treatment of cultured astroglia with Epi. We found, however, that the $\alpha_2$-AR agonist clonidine inhibited FSK-stimulated cAMP levels to the same extent in cells pretreated with Epi and in cells which were not treated with Epi. The reasons for this lack of change are unclear. Perhaps the downregulation of $\alpha_2A$-
AR in this system may not be reflected in a change in the cAMP pathway, but in some other signal transduction pathway. A variety of alternative signaling pathways have been associated with \( \alpha_2 \)-AR stimulation, including activation of potassium channels (Aghajanian and VanDerMaelen, 1982), inhibition of calcium channels (Holz et al., 1986), mobilization of intracellular calcium stores (Michel et al., 1989), and stimulation of PI hydrolysis (Wilson and Minneman, 1991; Cotecchia et al., 1990). Several of these mechanisms have been shown to operate in cultured astroglia (Enkvist et al., 1996). Perhaps the number of \( \alpha_2 \)-AR remaining after downregulation is adequate to elicit the observed suppression of cAMP accumulation, suggesting that a large receptor reserve exists. In addition, the limiting factor for \( \alpha_2 \)-AR-mediated inhibition of adenylyl cyclase may not be the receptor, but the amount of \( G_i \) (Neubig et al., 1985). In some cell systems, treatment with agents that increase cellular cAMP leads to increased expression of \( G_i \) (Hadcock et al., 1990), and therefore sensitization of the inhibitory pathway associated with \( \alpha_2 \)-AR. Another explanation for this observation in Epi-pretreated cultured astroglia is that clonidine is acting via so-called imidazoline receptors. However, the signal transduction mechanisms associated with imidazoline sites are uncertain (Regunathan and Reis, 1996), but are suggested to not involve cAMP (Regunathan et al., 1991). Therefore, it is unlikely that these sites are involved in the increased responsiveness to clonidine observed after Epi-pretreatment.

Our results indicate that \( \alpha_2 \)-AR expression is regulated in a heterologous manner by Epi via \( \beta \)-AR and \( \alpha_1 \)-AR. These results are novel in that they not only imply a negative regulatory role of cyclic AMP (via \( \beta \)-AR) on \( \alpha_2 \)-AR expression, by also a negative effect...
of PKC (via $\alpha_1$-AR). In addition, homologous regulation of $\alpha_{2A}$-AR also appears to occur in this system. These findings are summarized on Figure 5-6 and raise the question of the commonality of receptor regulation via several pathways. Such regulation could be a means whereby a cell can integrate a variety of signals and adjust its receptor complement accordingly. This type of regulation could also account for the apparent cell- or tissue-specific regulation of receptors. These findings suggest that regulation of $\alpha_{2A}$-AR expression is a complex process involving integration of multiple extracellular signals via several intracellular pathways. Integration of these pathways can lead to post-translational changes in receptor sensitivity or degradation as well as changes in de novo synthesis of receptors. It remains to be determined whether this is a universal effect true for other receptor families which interact with multiple signal transduction systems such as muscarinic, dopaminergic or somatostatin receptor families (Gudermann et al., 1996).
Figure 5-1. Effect of FSK or PMA on binding of $[^3H]$-MK 912 to membranes from cultured astroglia as a function of treatment time. Cultured astroglia were incubated with control solution (plotted on the y-axis; 0.5% DMSO in water), 10 µM FSK (●), or 500 nM PMA (■) for the indicated times. Specific binding was determined from the difference between $[^3H]$-MK 912 (0.2 nM) binding in the presence or absence of 100 µM Epi, and is expressed as a percent of control values (100%). Data are means ± SE from 3 independent experiments.
Figure 5-2. Effect of FSK or PMA on binding of [3H]-MK 912 to membranes from cultured astroglia as a function of [3H]-MK 912 concentration. Cultured astroglia were incubated with control solution, 10 µM FSK, or 500 nM PMA for 24 h. Saturation binding experiments were carried out as detailed in Materials and Methods. (A) Representative saturation curves showing specific binding of [3H]-MK 912 to membranes prepared from control (○), FSK treated (■), or PMA treated (▲) cultured astroglia. Specific binding was determined from the difference between binding in the absence or presence of 100 µM Epi. (B) The above specific binding data were analyzed according to the method of Scatchard (1949) to determine the apparent dissociation constant (Kd) for [3H]-MK 912 binding and the maximum number of binding sites (Bmax). Data are the mean of triplicate determinations. This experiment was repeated 4 times with similar results (Table 5-1).
Figure 5-3. Effect of Epi on binding of \[^{3}\text{H}\]-MK 912 to membranes from cultured astroglia as a function of \[^{3}\text{H}\]-MK 912 concentration. Cultured astroglia were incubated with control solution (100 µM ascorbate in 1X PBS) or 10 µM Epi for 24 h. Saturation binding experiments were carried out as detailed in Materials and Methods. (A) Representative saturation curve showing specific binding of \[^{3}\text{H}\]-MK 912 to membranes prepared from control (○) or Epi treated (■) cultured astroglia. Specific binding was determined from the difference between binding in the absence or presence of 100 µM Epi. (B) The above specific binding data were analyzed according to the method of Scatchard (1949) to determine the apparent dissociation constant (K_d) for \[^{3}\text{H}\]-MK 912 binding and the maximum number of binding sites (B_max). Data are the mean of triplicate determinations. This experiment was repeated 4 times with similar results (Table 5-1).
Figure 5-4. Effect of combined FSK and PMA treatment on binding of [³H]-MK 912 to membranes from cultured astroglia as a function of [³H]-MK 912 concentration. Cultured astroglia were incubated with control solution (1% DMSO), FSK (10 µM), or PMA (500 nM) for 24 h. Saturation binding experiments were carried out as detailed in Materials and Methods. (A) Representative saturation curves showing specific binding of [³H]-MK 912 to membranes prepared from control (■), FSK treated ( ), or PMA treated (▲) cultured astroglia. Specific binding was determined from the difference between binding in the absence or presence of 100 µM Epi. (B) The above specific binding data were analyzed according to the method of Scatchard (1949) to determine the apparent dissociation constant (K_D) for [³H]-MK 912 binding and the maximum number of binding sites (B_max). Data are the mean of triplicate determinations. This experiment was repeated 2 times with similar results.
Figure 5-5. Effect of 24 hr Epi pretreatment on ability of clonidine to inhibit cyclic AMP accumulation. Cultured astroglia were incubated with control solution or Epi (10 µM) for 24 h at 37°C. Following this the media were removed and replaced with serum-free media containing the indicated concentrations of clonidine. After two minutes the cells were treated with control solution (0.05% DMSO) or FSK (10 µM) for 20 min at 37°C. Following the incubations with FSK or control solution, the media were removed and cAMP was extracted and analyzed as detailed in Chapter 2. FSK-stimulated levels of cAMP in cultured astroglia pretreated with control solution (●) or Epi (■). Values are the means ± SE of 4 experiments. * significantly different from FSK-stimulated value.
CHAPTER 6  
CONCLUSIONS AND SUMMARY

Because a given cell may be exposed to a variety of hormonal or neurotransmitter inputs, it is not unreasonable to expect that mechanisms have evolved which allow the cell to adjust to those inputs. Cells employ a variety of receptor-effector mechanisms by which extracellular signals are transduced across the plasma membrane to produce intracellular chemical signals. Integration of these signaling pathways within the cell will determine how the cell responds and adjusts to differing physiological situations. Altering the regulatory status of hormone or neurotransmitter receptors and their signaling pathways represents one means whereby a cell can adjust to a variety of signals. Such regulation allows for integration of many pathways to adjust cellular responsiveness.

In this study we have investigated the regulation of $\alpha_{2A}$-AR in cultured astroglia. Few studies have examined the regulation of $\alpha_{2A}$-AR in non-transfected, non-transformed cells, and even fewer have explored $\alpha_{2A}$-AR regulation in cells derived from the CNS. Recent studies implicating $\alpha_{2A}$-AR in cardiovascular control (MacMillan et al., 1996) underscore the importance of understanding the basic cellular mechanisms involved in regulation of these receptors. Therefore, the primary aim of this study was to delineate cellular mechanisms involved in regulation of $\alpha_{2A}$-AR in astroglia cultured from the mammalian CNS.
During the course of our studies, FSK and PMA were observed to cause a rapid, but reversible, stellation of cultured astroglia. Elevations of cellular cAMP have been shown to alter astroglial morphology (Narumi et al., 1978). Changes in astroglial morphology have also been observed as a result of PKC activation (Arcuri et al., 1995). Because morphological changes have been associated with changes in astroglial gene expression (Yoshimura et al., 1996, Arcuri et al., 1995, Segovia et al., 1994), we wondered if the changes in α2A-AR expression we observed after FSK or PMA treatment were due to stellation of the cells. Treatment of cultured astroglia with Epi or Ang II produced decreases in levels of α2A-AR mRNA similar to those seen with FSK or PMA treatment, but without the changes in astroglial morphology. In addition, reversal of the morphological changes occurred over a faster time-frame than recovery of α2A-AR mRNA or number. Therefore, our results suggest that the downregulation of α2A-AR after FSK or PMA treatment are not associated with stellation of cultured astroglia.

The results of this study demonstrate that α2A-AR are regulated in a heterologous fashion in non-transfected, non-transformed cells. Heterologous downregulation had previously been described in cultured astroglia as a result of insulin treatment (Richards et al., 1987). However, the involvement of other intracellular pathways on heterologous regulation of α2A-AR in astroglia was virtually unknown. Most of our knowledge of α2A-AR regulation comes from studies in transformed cells. Studies in cell lines have focused on cAMP as an intracellular regulator of α2A-AR, because α2A-AR are coupled to inhibition of adenylyl cyclase. Only passing reference has been made to the lack of involvement of phorbol ester-stimulated PKC activity on α2A-AR regulation (Sakaue and
Hoffman, 1991; Hamamdzic et al., 1995). Cyclic AMP has been shown to increase $\alpha_{2A}$-AR expression in HT29 cells via a PKA-mediated decrease in transcription rate. Conversely, in astroglia, cAMP leads to decreased expression of $\alpha_{2A}$-AR via decrease in transcription rate. Similar decreases in expression were seen with activation of PKC. The different regulatory effects in HT29 cells and cultured astroglia after activation of these intracellular messengers may be due to differing cell lineage, cell culture conditions or cell transformation.

The results with cAMP accumulation and PKC activation led to the novel finding that ligands which can stimulate these pathways also cause decreased expression of $\alpha_{2A}$-AR. We found that Epi, via $\alpha_1$-AR and $\beta$-AR, decreases steady-state levels of $\alpha_{2A}$-AR mRNA. Similar decreases in levels of $\alpha_{2A}$-AR mRNA were observed via stimulation of AT$_1$ receptors with Ang II. These results implied activation of cAMP and PKC-mediated pathways because Epi can stimulate $\beta$-AR to cause cAMP accumulation and $\alpha_1$-AR to result in PKC activation (Bylund et al., 1994). Likewise stimulation of AT$_1$ receptors leads to activation of PKC (Sumners et al., 1996). The Epi-mediated effect on levels of $\alpha_{2A}$-AR mRNA also resulted in a decrease in the number of $\alpha_{2A}$-AR, although this decrease was much larger than that observed with FSK or PMA treatments. These results suggest that $\alpha_{2A}$-AR undergo heterologous regulation by Epi via stimulation of $\alpha_1$-AR and $\beta$-AR, and their intracellular signaling pathways. The results also suggest that activation of $\alpha_1$-AR and $\beta$-AR does not exclusively regulate $\alpha_{2A}$-AR expression, but that stimulation of other receptors which activate PKC or cause cAMP accumulation may regulate expression of $\alpha_{2A}$-AR.
The Epi-induced decrease in the number of $\alpha_{2A}$-AR did not appear to be due entirely to cAMP accumulation or PKC activation, because the decreases were much larger than those observed as a result of activation of either pathway alone. Simultaneous activation of cAMP and PKC pathways also did not elicit the same magnitude decrease as that produced by Epi treatment. This suggested that Epi was acting through mechanisms other than heterologous activation of intracellular pathways.

Epi does not appear to have homologous control of $\alpha_{2A}$-AR gene transcription in cultured astroglia, because Epi-induced decreases in steady-state levels of $\alpha_{2A}$-AR mRNA appear to be mediated by $\alpha_1$-AR and $\beta$-AR. Because the changes in $\alpha_{2A}$-AR number elicited by Epi cannot be mimicked by activation of pathways associated with the stimulation of $\alpha_1$-AR and $\beta$-AR, it is possible that post-translational homologous regulation may play a role in the Epi-induced decrease in $\alpha_{2A}$-AR number. Agonist-induced downregulation of $\alpha_{2A}$-AR number has been demonstrated in a variety of cell models. Further studies are needed to elucidate the contributions of the various adrenergic receptors to $\alpha_{2A}$-AR regulation.

In addition to the homologous and heterologous regulation exerted by Epi, we also questioned whether other ligands may exert heterologous regulation of $\alpha_{2A}$-AR. We found that Ang II via AT$_1$ receptors can promote heterologous downregulation of levels of $\alpha_{2A}$-AR mRNA. A similar antagonistic interaction between AT$_1$ receptors and $\alpha_2$-AR has been demonstrated in the nucleus tractus solitarius (NTS) of Sprague-Dawley and Wistar-Kyoto rats (Fior et al., 1994, 1995). Ang II immunoreactive nerve terminals have been shown to be colocalized with adrenergic and noradrenergic nerve terminals in the
NTS and in other cardiovascular control centers (Coveñas et al., 1990; Lind et al., 1985). In addition, AT$_1$ receptors and $\alpha_2$-AR are present in high density in the NTS (Tsutsumi and Saavedra, 1991; Rosin et al., 1993). This colocalization of neurotransmitters and receptors, along with evidence that Ang II decreases expression of $\alpha_2$-AR suggests that perturbations in the brain renin-angiotensin system may decrease $\alpha_2$-AR-mediated vasodepressor responses. However, in the brains of spontaneously hypertensive rats, Ang II enhances $\alpha_2$-AR affinity and function (Fior et al., 1995), suggesting a compensatory mechanism for controlling elevated blood pressure. Further studies on the interaction between Ang II and $\alpha_{2A}$-AR will be interesting to pursue in light of their roles in CNS control of blood pressure.

In addition to Ang II, other neuropeptides and their receptors are often colocalized with catecholaminergic neurons. Neuropeptide Y (NPY) is one such peptide which coexists with catecholamines in some neurons (Chronwall et al., 1985; Everitt et al., 1984). Within the NTS receptors for NPY and $\alpha_2$-agonists have been shown to be regulated in a reciprocal fashion; NPY decreased affinity and number of $\alpha_2$-AR, while the $\alpha_2$-AR agonist clonidine decreased affinity and number of NPY binding sites (Härfstrand et al., 1989). This change has been shown to be pertussis toxin sensitive (Von Euler et al., 1989), suggesting that these receptors may induce heterologous regulation of the other via a common $G_i$-protein. In fact, the hypotensive responses of NPY and $\alpha_2$-AR agonists are mediated via $G_i$ (Fuxe et al., 1989). These results in vivo combined with our in vitro results suggest that a variety of factors may interact to effect steady-state levels of $\alpha_{2A}$-AR, and hence functional responses to $\alpha_2$-AR agonists.
The large decreases we observed in number of $\alpha_{2A}$-AR after Epi treatment led us to believe we would observe an attenuation of the ability of $\alpha_{2A}$-AR agonists to inhibit cAMP synthesis. Instead, we observed that Epi-pretreatment did not change the ability of the agonist clonidine to inhibit cAMP accumulation. Similarly, in a previous study $\alpha_2$-AR responsiveness on cultured astroglia was not significantly attenuated after 18 h NE pretreatment, while $\beta$-AR responses were abolished (Atkinson and Minnemann, 1992). In cultured astroglia $\alpha_{2A}$-AR are coupled to a variety of effector pathways (Enkvist et al., 1996), some of which may be effected by $\alpha_{2A}$-AR downregulation and some which may not. Agonists for $\alpha_2$-AR produce a depolarizing effect on cultured astroglia (Hösli et al., 1982). The agonist clonidine also could produce increases in intracellular calcium (Salm and McCarthy, 1990; Zhao et al., 1992). This increase in calcium levels was shown to result from activation of phospholipase C and subsequent inositol trisphosphate accumulation (Enkvist et al., 1996). In addition, the levels of $G_i$ have been shown to increase in transformed cells after treatment with agents that increase cellular cAMP (Hadcock et al., 1990). It is possible that the lack of change in the response after Epi-pretreatment is due to tighter coupling of the remaining $\alpha_{2A}$-AR to inhibition of adenylyl cyclase because of an increase in the expression of $G_i$ (Neubig et al., 1985). In addition, the density of $\alpha_{2A}$-AR on cultured astroglia is approximately ten times higher (2-3 pmol/mg protein) than in transformed cells (110-380 fmol/mg protein; Sakaue and Hoffman, 1991; Devedjian et al., 1991; Hamamdzic et al., 1995). Even upon downregulation by Epi, $\alpha_{2A}$-AR number (~500 fmol/mg protein) in cultured astroglia exceeded that observed in transformed cells, suggesting that in cultured astroglia a large
receptor reserve may exist. The involvement of these alternative signaling pathways in 
$\alpha_{2A}$-AR expression and function will be exciting to explore.

This study led us to question the source of catecholamines which may influence 
$\alpha_{2A}$-AR on astroglia in vivo. It is unlikely that circulating catecholamines can effect 
astroglia because the blood-brain barrier formed by brain endothelial cells does not allow 
passage of hydrophilic substances (Crone and Olesen, 1982; Butt et al., 1990). However, 
release of Epi or NE from catecholaminergic neurons can effect astroglial function in vivo 
either by direct synaptic contact (Vernadakis, 1988) or by non-synaptic release (Chiu and 
Krieger, 1994). In addition, ascending catecholaminergic fibers from brainstem nuclei 
such as the locus ceruleus are unique in that many of them do not terminate in synaptic 
contacts, but in varicosities (Beaudet and Descarries, 1984), from which catecholamines 
diffuse to their targets (Dismukes, 1977). Astroglia have been proposed to be a major 
target of such innervation (Stone and Ariano, 1989).

One of the best known physiological effects of adrenergic stimulation of astroglia 
is glycogenolysis (Cummins et al., 1983a, 1983b; Pearce et al., 1985; Cambray-Deakin et 
al., 1988). The finding that activation of both $\alpha_{2}$-AR and $\beta$-AR contribute to increases in 
astroglial glycogenolysis (Subbarao and Hertz, 1990) suggests that these receptors play a 
metabolically important role in CNS physiology. Studies have shown that astroglial 
glycogenolysis can produce lactate (Dringen et al. 1993; McKenna et al., 1993) and that 
lactate can be used by neurons as an energy source (Larrabee, 1983, 1992; Schurr et al., 
1988). Direct evidence has also been provided that lactate released from astroglia is 
utilized by nearby neurons (Poitry-Yamate et al., 1995). Lactate release appears to occur
via neurotransmitter-mediated processes (Pellerin and Magistretti, 1994; Poitry-Yamate et al., 1995; Elekes et al., 1996) suggesting that glial energy reserves (Ibrahim, 1975) are accessed by neurons in a use-dependent manner. Perhaps the lack of change of responsiveness to $\alpha_2$-AR agonists in cultured astroglia despite decreased $\alpha_2A$-AR number is a mechanism designed to maintain the metabolic relationship between neurons and astroglia in the face of sustained neurotransmitter challenge. However, continued downregulation of $\alpha_2A$-AR may lead to perturbations in the metabolic relationship.

Alterations in the metabolic relationship between neurons and astroglia has previously been implicated in affective disorders and Alzheimer's disease (Hertz and Richardson, 1983; Hertz, 1989). The recent advances in our understanding of the metabolic and functional coupling of neurons and astroglia suggest the importance of studying regulation of neurotransmitter-mediated events on astroglia.

The findings of the present study are summarized on Figure 6-1. The results of this study have shown that $\alpha_2A$-AR in cultured astroglia are regulated by the intracellular signaling messengers cyclic AMP and PKC. Increases in cyclic AMP accumulation or PKC activation lead to decreased transcription of the $\alpha_2A$-AR gene and subsequently, decreased steady-state levels of $\alpha_2A$-AR mRNA. These cAMP or PKC-mediated decreases in levels of $\alpha_2A$-AR mRNA lead to decreased expression of $\alpha_2A$-AR protein. These results suggested that endogenous ligands which increase cAMP accumulation or PKC activation could lead to similar changes in $\alpha_2A$-AR expression. Treatment of cultured astroglia with Epi resulted in decreased steady-state levels of $\alpha_2A$-AR mRNA via stimulation of $\alpha_1$-AR and $\beta$-AR, receptors which are coupled to PKC activation and
cAMP accumulation, respectively (Bylund et al., 1994). Epi-treatment also led to decreased expression of $\alpha_{2A}$-AR protein. Ang II treatment also caused decreases in levels of $\alpha_{2A}$-AR mRNA. These decreases were mediated by AT$_1$ receptors which have been shown to couple to PI hydrolysis and subsequent activation of PKC (Sumners et al., 1994, 1996).
Figure 6-1. Summary of processes involved in the regulation of $\alpha_{2A}$-AR in cultured astroglia. Dashed lines indicate a downregulatory effect.
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BIOGRAPHICAL SKETCH

Michael A. Reutter was born on October 22, 1959, in Rochester, Minnesota. He received his Bachelor of Arts degree from Gustavus Adolphus College in 1982 with a double major in biology and general sciences. In 1985, he graduated from Florida State University with a Master of Science degree in biological oceanography. After working for the State of Florida, Department of Environmental Regulation for seven years he entered the University of Florida, Department of Physiology, and chose to work in the laboratory of Dr. Colin Sumners. Upon arrival he received a three year award as a College of Medicine Scholar. He was also awarded predoctoral fellowships from the University of Florida, Center for Neurobiological Sciences (1993-1994), and from the American Heart Association, Florida Affiliate (1994-1996). He has been an active participant in the Gainesville Civic Chorus and the Jacksonville Symphony Chorus. When he completes the requirements for his Ph.D. he will begin postdoctoral work at the University of Minnesota, Department of Cell Biology and Neuroanatomy, in the laboratory of Dr. Virginia Seybold.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Physiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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