AN ABSTRACT OF THE THESIS OF

Barbara D. Hettinger for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on August 26, 1997. Title: Regulation of Neuronal A1 Adenosine Receptors

Abstract approved:

Thomas F. Murray

Adenosine is a ubiquitous physiological regulator and neuromodulator which exerts its actions in multiple tissues through activation of receptors which are members of the superfamily of G-protein coupled receptors. Regulation of G-protein coupled receptors, G-proteins and the associated effectors provides a means for cells to adapt to chronic agonist or antagonist treatment and therefore to maintain homeostasis. Primary cultures of cerebellar granule cells were utilized as a model for neuronal A1 adenosine receptor (A1AR) regulation. Chronic exposure to the A1AR agonist, N6-cyclopentyladenosine resulted in: (1) a time- and concentration-dependent reduction in the density of receptors labeled by the A1AR selective antagonist [3H]1,3-dipropyl-8-cyclopentylxanthine, (2) an enhanced ability of guanyl nucleotides to decrease the fraction of A1AR sites displaying high affinity for 2-chloroadenosine, (3) a functional uncoupling of receptors from adenylyl cyclase which was homologous in nature and (4) sequestration of A1AR. The time course of the agonist-induced sequestration was much slower than that reported for other G-protein coupled receptors. Steady state A1 adenosine receptor mRNA levels as well as transcript stability were unaffected by exposure to agonist for 48 hours. We have determined that the half-life of A1AR mRNA in cerebellar granule cells is 21 hours which is considerably longer than that reported for other G-protein coupled receptors. The slow time
course of A$_1$AR sequestration and stability of its message may be a reflection of the tonic inhibitory tone exerted by adenosine.

The adenosine antagonists caffeine and 8-parasulfophenyltheophylline produced alterations in A$_1$AR homeostasis which were antipodal to those associated with agonist treatment. Antagonist exposure: (1) increased the density of A$_1$AR in cerebellar granule cell membranes, (2) blunted the effect of guanyl nucleotides on receptor coupling to G-proteins, and (3) increased the functional coupling of receptors to adenylyl cyclase inhibition. Forskolin treatment of cerebellar granule cells did not affect receptor density suggesting that cAMP is not involved in the regulation of A$_1$AR expression. There was no effect of 8-pSPT treatment or removal of endogenous adenosine by adenosine deaminase on A$_1$AR sequestration indicating that A$_1$AR sequestration is not affected by tonic levels of adenosine.
Regulation of Neuronal A₁ Adenosine Receptors

by

Barbara D. Hettinger

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Barbara D. Hettinger, Author
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CONTRIBUTION OF AUTHORS

Dr. Thomas F. Murray is the principal investigator of the project and was involved in the design, analysis and writing of each manuscript. Dr. Mark Leid was also involved in the design and analysis of each manuscript.
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Regulation Of Neuronal A₁ Adenosine Receptors

Chapter 1

Introduction

1.1. Physiological Actions of Adenosine

In 1929, Drury and Szent-Gyorgyi presented the first evidence that exogenous adenosine application produced a marked hypotension and decreased heart rate, as well as affecting kidney function (Drury and Szent-Gyorgyi, 1929). Since that time, adenosine has been demonstrated to have diverse effects in a number of tissues and cell types including renal, fat, pulmonary, cardiac and immune, as well as the central nervous system.

In adipocytes, adenosine inhibits lipolysis and stimulates glucose uptake (reviewed in LaNoue and Martin, 1994). A role for adenosine in obesity has been proposed by LaNoue and Martin (1994) in that there is abnormal adenosine function in Zucker rat (fa/fa) and obese mouse (ob/ob) models of obesity. This group has hypothesized that adenosine signaling in mutant rodents is unusually and tonically active. Metabolic effects of adenosine may also play a role in diabetes mellitus (Barrington, et al., 1996) in that induction of diabetes mellitus reduces the ability of adenosine to inhibit lipolysis which may potentially worsen hyperlipidemia associated with this syndrome.

A role for adenosine in the immune system has recently been demonstrated in several cell types. Adenosine acts to potentiate release of mediators of the immediate hypersensitivity response from mast cells, including leukotriene C₄ and histamine (reviewed by Linden, 1994). In neutrophils, elevated adenosine levels in response to ischemic damage can inhibit both the generation of harmful superoxide metabolites (Cronstein, 1994) and adherence of neutrophils to endothelial cells (reviewed by
Fredholm, 1997). Basal endogenous levels of adenosine may promote neutrophil chemotaxis and adherence to endothelial cells via a different adenosine receptor subtype which is tonically activate (Cronstein, 1994). Adenosine has also been implicated in allergic asthma: in healthy individuals, adenosine acts as a bronchodilator, whereas in asthmatics adenosine produces bronchoconstriction (Cushley, et al., 1983 and reviewed by Holgate, et al., 1987 and Linden, 1994). The degranulation of mast cells within the pulmonary system and subsequent release of histamine and leukotriene C₄ act to potentiate the action of allergens leading to constriction of pulmonary vascular beds and increased pulmonary resistance. Recently, inhalants containing adenosine receptor (A₁ subtype) antisense oligonucleotides were evaluated in an rabbit allergic asthma model. Nyce and Metzger (1997) demonstrated a reduction of airway constriction in rabbits receiving antisense oligonucleotide treatment indicating the involvement of adenosine in asthma.

The cardiodepressive effects of adenosine are manifold and adenosine and related analogs as well as inhibitors of adenosine transport and metabolism have been evaluated for therapeutic potential. Adenosine produces negative chronotropic, dromotropic and inotropic effects in the heart which reflects the broad distribution of adenosine receptors in cardiac tissue (reviewed in Olsson and Pearson, 1990). The negative inotropic effects of adenosine are distinct in atrial and ventricular chambers. In atria adenosine acts directly to shorten the duration of the cardiac action potential due to an increase in potassium conductance of atrial muscle. The shortened action potential duration limits calcium influx which in turn decreases the force of contraction (Olsson and Pearson, 1990). Adenosine has neither of these effects in ventricles. Adenosine in both atria and ventricles acts indirectly to inhibit the stimulatory effects of adrenergic receptor activation (Olsson and Pearson, 1990). Other inhibitory effects of adenosine on cardiac tissue include dilation of cardiac vascular beds (Collis and Hourani, 1993) and inhibition of platelet aggregation (Paul, et al., 1990). Adenosine and its
analogs have been exploited clinically to treat tachycardias, although adverse effects may include adenosine-induced hypotension (Barber, 1992). Adenosine may play a role in preconditioning of cardiac tissue in which an initial ischemic event can protect against subsequent ischemic injury (Liu, et al., 1991 and 1994; Armstrong and Ganote, 1994 and 1995; Rice et al., 1996). The initial preconditioning ischemic event causes release of adenosine which then acts to limit the excitatory damage caused by the second event. A recent study by Matherne, et al. (1997) has demonstrated ischemic protection and improved functional recovery time in mice overexpressing the A₁ adenosine receptor subtype. The authors suggest that cardiac adenosine receptors are saturated by endogenous adenosine in wild type animals which precludes the ability of adenosine analogs to provide additional protection (Matherne, et al., 1997).

In the kidney, adenosine acts as an antidiuretic by modulation of renal blood flow including vasoconstriction of afferent arterioles and vasodilation of efferent arterioles which collectively result in decreased glomerular filtration rate (Spielman, et al., 1980). Adenosine also mediates inhibition of renin release which results in a decrease in blood pressure (Spielman and Thompson, 1982).

In the central nervous system, endogenous adenosine (approximately 50-300 nM in brain, Parkinson, et al., 1994) has a neuroprotective role via inhibition of excitatory neurotransmission at pre- and postsynaptic targets (reviewed by Rudolph, et al., 1992; Parkinson, et al., 1994; Fredholm, 1995; Sweeney, 1997). Adenosine acts at presynaptic sites to inhibit release of excitatory neurotransmitters such as glutamate and acts at postsynaptic sites to hyperpolarize target cells (Fredholm and Dunwiddee, 1988; Fredholm, et al., 1993). Adenosine also acts to increase cerebral blood flow via dilation of cerebral blood vessels. Other effects of adenosine in the CNS include sedation, which may be important for the onset and maintenance of sleep (Huston, et al., 1996), attenuated locomotor activity and decreased alertness
(Fredholm, et al., 1993). In the spinal cord, adenosine is likely to modulate sensory neurotransmission and play a role in analgesia. Adenosine agonists have been shown to have antiepileptic properties in various seizure paradigms and it has been proposed that adenosine acts as an endogenous anticonvulsant (reviewed by Knutsen and Murray, 1997). Adenosine analogs and/or agents that can modulate the local concentration of adenosine are also able to reduce the injury associated with stroke providing further evidence that adenosine may act as an endogenous neuroprotectant (Parkinson, et al., 1994; Sweeney, 1997). Adenosine, acting to inhibit excitation in the brain may play critical roles in neurodegenerative disorders such as Alzheimer's and Parkinson's disease which may involve toxicity due to the excess excitatory amino acid release (Daval, et al., 1991). Thus, the potential therapeutic uses of adenosine analogs are manifold and an understanding of adenosinergic signal transduction is critical to future attempts to manipulate adenosine receptor signaling for therapeutic advantage.

1.2. Adenosine Receptors

1.2.1. Characterization Of Adenosine Receptors

Adenosine acts at receptors that are members of the superfamily of G-protein coupled receptors (GPCR) which have seven transmembrane spanning domains and couple to their effectors via guanyl nucleotide binding proteins (G-proteins). Four adenosine receptors have been characterized initially by distinctive pharmacological profiles and later by sequence information (reviewed by Olah and Stiles, 1995). The A_1 adenosine receptor (A_1AR) has been cloned from rat, human, dog, cow and rabbit and ranges in size from 326 to 328 amino acids (Libert, et al., 1991; Mahan, et al., 1991; Reppert, et al., 1991; Libert, et al., 1992; Olah, et al., 1992; Townsend-Nicholson and Shine, 1992; Tucker, et al., 1992; Bhattacharya, et al., 1993; Ren and Stiles, 1994). The A_2AAR ranges in size from 409-412 amino acids
and is the largest adenosine receptor due primarily to an extended carboxyl terminal intracellular tail (Libert, et al., 1991; Fink, et al., 1992; Furlong, et al., 1992; Rivkees and Reppert, 1992; Marquardt, et al., 1994). The A_{2B}AR is 328-332 amino acids (Pierce, et al., 1992; Stehle, et al., 1992) and the most recently discovered A_{3}AR is 317-320 amino acids in length (Zhou, et al., 1992; Linden, et al., 1993; Salvatore, et al., 1993). The initial agonist rank order potency demonstrated for the A_{1}AR was R-phenylisopropyl-adenosine (R-PIA) > 5'-N-ethylcarboxamide adenosine (NECA) > S-phenylisopropyladenosine (S-PIA). The bovine A_{1}AR however exhibits a distinct pharmacological profile (R-PIA > S-PIA > NECA) with approximately 10-fold higher affinity for both agonists and antagonists compared to rat A_{1}AR although the cDNA sequences of bovine and rat A_{1}AR are greater than 90% identical (Olah, et al., 1992; Tucker, et al., 1992). A_{2}ARs were classified by the agonist potency profile NECA > R-PIA > S-PIA with A_{2A}AR and A_{2B}AR representing high and low affinity agonist binding, respectively. A_{3}AR displays the agonist potency profile NECA > R-PIA > S-PIA (reviewed by Palmer and Stiles, 1995).

Adenosine receptors are ubiquitous in mammalian tissues although each subtype has a distinct localization pattern. Expression of A_{1}AR mRNA is high in brain and is particularly abundant in the cortex, cerebellum, thalamus and hippocampus (Stehle, et al., 1992). A_{1}AR mRNA has also been detected in spinal cord, fat, testis, heart and kidney (Mahan, et al., 1991; Reppert, et al., 1991; Olah, et al., 1992; Stehle, et al., 1992). Ren and Stiles (1995) demonstrated that the human A_{1}AR utilizes two separate promoters to direct synthesis of distinct transcripts which may regulate the relative abundance of A_{1}AR in various tissues. Expression of transcript α initiated by promoter A was demonstrated in tissues with high A_{1}AR expression, including brain (frontal cortex and cerebellum), testis and kidney. Transcript β initiated by promoter B was found to be expressed in all tissues investigated, although at lower levels (Ren and Stiles, 1994). The localization of A_{2A}AR is more restricted in the brain with abundant message in the striatum, smaller amounts
in the cortex and midbrain and no detectable message in the hypothalamus or cerebellum (Stehle, et al., 1992). Interestingly, in the brain, the A$_{2A}$AR colocalizes with dopamine D$_2$ receptor mRNA on subsets of striatal neurons indicating a functional link between these two pathways (Fink, et al., 1992). A$_{2A}$AR mRNA has also been detected in the heart, kidney and lung and, pharmacologically, these receptors have been demonstrated in liver and on platelets. The A$_{2B}$AR is present in the cecum, large intestine and urinary bladder with lower amounts in brain, spinal cord, lung and mast cells from mouse bone marrow (Stehle, et al., 1992). The tissue distribution of A$_3$AR mRNA displays the greatest degree of species-specific differences but is present in lung of all species. In rat, A$_3$AR is also present in kidney and heart in moderate levels with lower expression in cortex and striatum (Zhou, et al., 1992; Linden, et al., 1993; Salvatore, et al., 1993). In human the A$_3$AR is highest in lung and liver followed in order of abundance by brain, aorta, testis and heart (Salvatore, et al., 1993).

Adenosine receptors share several structural motifs common to all GPCR. Each of the cloned adenosine receptor subtypes has potential N-linked glycosylation sites and the A$_1$AR and A$_{2A}$AR have been identified as glycoproteins in native tissue (Stiles, 1986; Barrington, et al., 1990; Palmer, et al., 1992; Pierson, 1994; reviewed in Palmer and Stiles, 1995). The glycosylation sites for A$_1$AR and A$_{2A}$AR are on found in the second extracellular loop. The A$_3$AR has potential glycosylation sites at the amino terminus. The functional significance of adenosine receptor glycosylation has yet to be determined. Another motif that the adenosine receptors share with the larger family of GPCR is an aspartate in the second transmembrane spanning domain which may be required for regulation of agonist binding by sodium ions. Cysteines, present in adenosine receptor extracellular loops and those of other GPCR may be required for disulfide bond formation which may be critical for the conformational integrity of the receptor (Jacobson, et al., 1992). A conserved cysteine in the carboxyl tail of A$_1$AR, A$_{2B}$AR and A$_3$AR
may be important for palmitoylation of these receptors (Parsons and Stiles, 1995).

Adenosine receptors modulate the activity of a number of effectors via activation of specific G-proteins. A₁AR appears to couple specifically to members of the group of inhibitory G-proteins with a selectivity for recombinant α subunits of: Gβ3>α2=Gβ1=α (Freissmuth, et al., 1991). There was no coupling of the A₁AR to Gβ or Gα (Freissmuth, et al., 1991). The A₂ₐAR and A₂₈AR preferentially couple to the stimulatory G-protein, Gα whereas A₃AR couples to at least Gα2 and Gα3, and possibly other inhibitory G-proteins (Zhou, et al., 1992, Linden, et al., 1993, Salvatore, et al. 1993 and Palmer and Stiles, 1995). The coupling of A₁AR and A₂ₐAR to their respective G-proteins has been reported to be tighter than coupling observed for other GPCR. Munshi and Linden (1989) demonstrated that receptor solubilization did not reduce high affinity agonist binding to A₁AR suggesting that G-proteins were tightly coupled to and copurified with A₁AR.

Activation of A₁AR at both presynaptic and postsynaptic sites produces effects at a number of downstream targets. Classically, A₁AR were reported to be coupled to inhibition of adenyl cyclase reducing intracellular levels of cAMP. More recently direct coupling of A₁AR to direct activation of potassium channels including inward rectifying acetylcholine sensitive and ATP-sensitive currents has been demonstrated in cardiac and neuronal cells. This activation of potassium channels results in hyperpolarization of target cells. A₁AR are also coupled to inactivation of calcium channels. A₁AR mediated inhibition of adenyl cyclase and subsequent decreases in intracellular cAMP levels result in the direct inactivation of calcium channels and hyperpolarization of cells indirectly inactivates voltage dependent calcium channels (Greene and Haas, 1991; Iredale, et al., 1994). Adenosine activation of A₁AR also inhibits chloride currents (Forrest, 1996). Activation of presynaptic A₁AR and modulation of calcium, potassium and chloride conductances act to prevent release of excitatory neurotransmitters. Other
effects of A$_2$AR activation include increased glucose transport, inactivation of phospholipase A$_2$ and either stimulation or inhibition of phospholipase C (Schiemann and Buxton, 1991; Akbar, et al., 1994) in a tissue dependent manner. A$_{2A}$AR and A$_{2B}$AR couple to the stimulation of adenylyl cyclase and increase in the intracellular cAMP whereas A$_3$AR inhibits adenylyl cyclase (Windschief, 1996).

The cloning of the four adenosine receptor subtypes from several species have prompted attempts to determine the structural requirements for ligand binding and G-protein coupling. A model has been proposed for the ligand binding pocket of catecholamine receptors, however, unlike catecholamines, adenosine is uncharged at physiological pH and is sterically bulkier than, e.g. epinephrine and may therefore have different structural requirements (Palmer and Stiles, 1995). A high degree of conservation between the adenosine receptor subtypes in transmembrane domains 2, 3 and 7 has been observed and proposed to represent a potential ligand binding pocket (Linden, et al., 1994). Several groups have demonstrated specific amino acid requirements for ligand binding. The importance of histidine residues in ligand binding was investigated by treatment of membranes with the histidine reactive compound diethylpyrocarbonate (DEPC) (Klotz, et al., 1988). DEPC treated membranes displayed reduced binding of both A$_1$AR and A$_{2A}$AR agonists and antagonists indicating an importance of histidine residues in ligand binding. Olah, et al. (1992) reported a reduction in both agonist and antagonist binding in a mutant A$_1$AR in which histidine 274 within the seventh transmembrane spanning domain was mutated to leucine. Another histidine to leucine mutation at amino acid 256 in transmembrane spanning domain six of A$_1$AR resulted in decreased antagonist binding with no change in agonist binding (Olah, et al., 1992). Site-directed mutagenesis has also demonstrated the importance of amino acids 270 and 277 (Tucker, et al., 1994). Threonine 277 was determined to be important for high affinity binding of 5' substituted agonists. The identity of
the amino acid at position 270 was determined to be responsible for species specific differences in binding of the N\textsuperscript{6}-substituted agonist R-PIA and the C\textsuperscript{8}-substituted antagonist DPCPX.

In addition to site-directed mutagenesis of individual amino acids, chimeric receptors have been utilized to define structural requirements for ligand binding of adenosine receptors. Experiments utilizing chimeras of A\textsubscript{1}AR and A\textsubscript{3}AR established that the region conferring high affinity for 5’ substituted agonists consisted of a six amino acid sequence within the fifth transmembrane spanning domain adjacent to the second extracellular loop (Olah, et al., 1994). In addition, the carboxyl terminal eleven amino acids of this loop in A\textsubscript{1}AR were determined to be important for binding of xanthine antagonists (Olah, et al., 1994). Therefore, Olah, et al. (1994) proposed that overlapping regions in the second extracellular loop were important for agonist and antagonist binding. Rivkees et al. (1995) have created A\textsubscript{1}AR/A\textsubscript{2A}AR chimeras and analysis of these mutants indicated that important determinants for A\textsubscript{1}AR agonist and antagonist binding as well as ligand specificity resided in transmembrane domains 1-4. Rivkees, et al. (1995) hypothesized that the N\textsuperscript{6}/C\textsuperscript{8} positions of adenosine agonists and antagonists, respectively, interact with transmembrane domains 1-4 and the 5’ ribose moiety interacts with transmembrane domain 7.

**1.2.2. Regulation Of Adenosine Receptors - Upregulation**

Regulation of G-protein coupled receptors in response to chronic agonist and antagonist exposure has both physiological and clinical relevance. The effects of chronic antagonism of adenosine receptors by the nonselective methylxanthines caffeine and theophylline is of particular interest as these compounds are ingested by humans throughout the world. Blockade of adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} adenosine receptors is the likely mechanism for the stimulatory effects of these compounds. In general, the A\textsubscript{3}AR are not as sensitive to the classical xanthine antagonists with some
exceptions. Chronic antagonism of adenosine receptors enhanced the response to subsequent adenosine analogs in several animal models. Chronic caffeine ingestion in rats increased the inhibitory action of adenosine in cerebral cortical neurons (Lin and Philis, 1990). Also in rats, chronically exposure to theophylline sensitized the indirect antiadrenergic effects of adenosine receptor agonists in cardiac tissue. Interestingly, in the same study, the direct negative chronotropic and inotropic effects of adenosine were not sensitized due to chronic theophylline exposure (Lee, et al., 1993b).

Several groups have investigated the effects of chronic caffeine and theophylline exposure on seizure susceptibility (Szot, et al., 1987; Sanders and Murray, 1989; Georgiev, et al., 1993) and ischemia (Rudolphi, et al., 1989). Long term antagonist ingestion reduced the susceptibility of mice to N-methyl-D-aspartate (NMDA) induced seizures (Georgiev, et al., 1993) and rats to bicuculline induced seizures (Szot, et al., 1987; Sanders and Murray, 1989), presumably via sensitization of adenosine receptor signaling. Long term caffeine exposure also protected gerbils from ischemia-induced neuronal damage (Rudolphi, et al., 1989).

In most of these studies, the physiological sensitization to adenosine analogs was accompanied by an upregulation of adenosine receptors as measured by radioligand binding (reviewed by Daly, 1993). Chronic methylxanthine exposure of rats produced an increased density of A1AR as measured by radioligand binding in membranes from several brain regions including cortex, cerebellum and hippocampus (Murray, 1982; Green and Stiles, 1986; Szot, et al., 1987; Zielke and Zielke, 1987; Ramkumar, et al., 1988; Sanders and Murray, 1989; Wu, et al., 1989; Shi, et al., 1993). Similarly, autoradiographic studies by two groups (Rudolphi, et al., 1989; Johannsson, et al., 1993) demonstrated an increase in A1AR binding in the rat CA3 (Johannson, et al., 1993) and gerbil CA1 (Rudolphi, et al., 1989) subfields of the hippocampus in response to chronic caffeine ingestion. In contrast, Fastbom and Fredholm (1990) did not observe a significant increase
in hippocampal A₃AR by autoradiography and suggested that instead there was an altered coupling between the A₃AR and G-proteins. In two studies, the increased density of receptors was accompanied by increases in the proportion of A₃AR in the agonist high affinity state and G,α subtype levels, as well as a sensitization of adenylyl cyclase to inhibition by adenosine analogs (Green and Stiles, 1986; Ramkumar, et al., 1988). The effects of chronic theophylline administration in rat cardiac tissue included upregulation of both atrial and ventricular A₃AR with no change in receptor affinity or receptor coupling to G-proteins (Lee, et al., 1993b). Zhang and Wells (1990) investigated the effects of chronic caffeine exposure on adipocyte and platelet adenosine receptors and found that platelets were sensitized to the effects of adenosine whereas adipocytes were not, although adipocytes did display and increase in A₃AR density. Methylxanthine-induced upregulation of A₃AR was not found to be associated with an increase in receptor mRNA (Johannson, et al., 1993) which indicates that A₃AR are not transcriptionally regulated.

Functional manifestations of these alterations in A₃AR in response to chronic antagonist exposure include an enhanced sensitivity of A₃AR agonist-induced maximal inhibition of adenylyl cyclase in rat cerebral cortical membranes (Green and Stiles, 1986; Ramkumar, et al., 1988) and guinea pig cardiomyocytes (Wu, et al., 1989) or an increase in basal and isoproterenol-stimulated cyclase in DDT, MF-2 cells (Stille and Stiles, 1991). Furthermore, Daval, et al. (1989) used binding of [³²P]forskolin to demonstrate that caffeine increased the density of adenylyl cyclase protein in rat brain slices. In contrast to these reports, Zhang and Wells (1990) indicated that there was no change in A₃AR mediated inhibition of adenylyl cyclase or lipolysis in rat adipocytes due to chronic caffeine ingestion. Holtzman, et al. (1991) also did not find a change in receptor-mediated inhibition of adenylyl cyclase in rat cerebral cortical membranes from rats chronically exposed to caffeine. These differences may be due to species and tissue specific differences in regulation of A₃AR.
The effect of chronic exposure to methylxanthines on adenosine A\textsubscript{2A} and A\textsubscript{2B} adenosine receptors has not been characterized to the same extent due to a delay in availability of compounds displaying selective binding to each subtype. A report by Zhang and Wells (1990) demonstrated an increased stimulatory effect of NECA on adenylyl cyclase in caffeine treated rat platelets and ascribed this to a possible increase in A\textsubscript{2A}AR and/or an enhanced coupling of these receptors to stimulatory G-proteins. However, Johansson, et al. (1993) and Shi, et al. (1993) reported that A\textsubscript{2A}AR did not appear to be upregulated in response to chronic methylxanthines.

In addition to antagonist-induced upregulation, adenosine receptor levels can be increased in response to exposure to glucocorticoids. Glucocorticoids exert their effects via activation of glucocorticoid receptors and regulation of the transcription of a number of genes (Truss and Beato, 1993). The synthetic glucocorticoid, dexamethasone was initially reported to alter the expression of the prototypical GPCR, the \( \beta \)-adrenergic receptor (Collins, et al., 1988). Two reports from Fredholm's group demonstrate that adenosine receptor expression can also be regulated by glucocorticoid receptor activation (Gerwins and Fredholm, 1991; Svenningsson and Fredholm, 1997). In DDT, MF-2 smooth muscle cells, glucocorticoid receptor activation by dexamethasone resulted in an 50% upregulation of A\textsubscript{1}AR with no change in receptor affinity or fraction of receptors in the high and low agonist affinity states (Gerwins and Fredholm, 1991). These results were specific to glucocorticoid receptor activation as other steroid hormones were much less effective at producing an increase in A\textsubscript{1}AR and addition of RU486, a glucocorticoid receptor antagonist, prevented the upregulation of A\textsubscript{1}AR. The translational inhibitor, cycloheximide also prevented upregulation indicating a requirement for new protein synthesis. Adenylyl cyclase activity was sensitized to the inhibitory effects mediated by A\textsubscript{1}AR although alterations in G-protein levels did not accompany the glucocorticoid-mediated upregulation. In the same study, A\textsubscript{2A}AR was downregulated and A\textsubscript{2B}AR- mediated stimulation of
adenylyl cyclase was decreased in response to dexamethasone treatment (Gerwins and Fredholm, 1991). In rat brain, glucocorticoids were found to regulate A₁AR but not A₂A₂AR expression (Svenningsson and Fredholm, 1997). Removal of glucocorticoid stimulation via adrenalectomy reduced A₁AR mRNA levels as well as binding of the A₁AR selective antagonist [³H]DPCPX and these effects were reversed by addition of dexamethasone. Dexamethasone did not have an effect on affinity or fraction of A₁AR in the high and low affinity states. In this study, glucocorticoids did not regulate the expression of A₂A₂AR in rat brain (Svenningsson and Fredholm, 1997) which may be due to tissue specific differences.

1.2.3. Regulation Of Adenosine Receptors - Downregulation

The ability of chronic antagonist exposure to upregulate and sensitize adenosine receptors suggests the presence of a tonic inhibition by endogenous adenosine that is relieved in the presence of antagonists. The presence of either endogenous or pathophysiological levels of adenosine may chronically downregulate and/or desensitize adenosine receptors. Several groups have investigated the response of adenosine receptors to chronic agonist exposure in adipocytes, cardiac tissue, brain and the smooth muscle cell line DDT₁, MF-2.

Initial studies of the effects of chronic adenosine receptor activation utilized adipocytes isolated from rats exposed to chronic agonist or cultured adipocytes exposed to adenosine agonists in vitro. Prolonged exposure to the A₁ selective adenosine receptor agonist, (+)-N⁶-(R-phenylisopropyl-adenosine) (R-PIA) altered the lipolytic response of adipocytes isolated from rat epididymal fat pads (Hoffman, 1986). The inhibitory effect on lipolysis by adenosine analogs was abolished and there was a decreased sensitivity to agonist-induced inhibition of adenylyl cyclase in adipocytes from rats infused with R-PIA. In a study by Green (1987), exposure of cultured adipocytes to R-PIA resulted in a similar decrease in the inhibition of lipolysis by R-PIA as
well as decreased sensitivity to insulin. The density of $A_r$AR in adipocytes was reduced by R-PIA treatment in vitro with no change in receptor affinity. R-PIA exposure also produced a decrease in the levels of inhibitory G-proteins. Parsons and Stiles (1987) confirmed these results for $A_r$AR in membranes derived from R-PIA treated rat adipocytes and demonstrated a reduction in $A_r$AR high affinity agonist binding sites. In addition to a decrease in $G_\alpha$-subunits, $G_g\alpha$ was demonstrated to increase in response to chronic adenosine receptor agonist exposure. The decreased sensitivity of $A_r$AR-mediated inhibition of adenylyl cyclase was shown to be heterologous, in that prostaglandin E₁ (PGE₁) inhibition of adenylyl cyclase was also attenuated (Parsons and Stiles, 1987; Green, et al., 1992). Longabaugh, et al. (1989) determined which of the inhibitory G-proteins were involved in the adipocyte response to chronic agonist exposure demonstrating a decrease in $G_\text{i}\alpha 1$ and $G_\alpha 2$ with no change in $G_\text{i}\alpha 3$ and increased $G_\text{g}\alpha$. These results are not consonant with those of Green, et al. (1990) who found an almost complete loss of $G_\text{i}\alpha 1$ and $G_\text{g}\alpha 3$ with a less dramatic effect on $G_\text{g}\alpha 2$ and $G_\beta$ in cultured adipocytes exposed to adenosine analogs.

Cardiac $A_r$AR have also been demonstrated to downregulate and lose sensitivity in response to chronic agonist exposure which is of particular importance in the development of adenosinergic treatments for ischemia. Embryonic chick heart exposed to the R-PIA in ovo for 44 hours demonstrated a decrease in $A_r$AR (Shyrock, et al., 1990). Shyrock, et al. (1990) demonstrated both a reduction in $A_r$AR binding sites and a blunted ability of R-PIA to decrease contractile tension in hearts from chicks chronically exposed to agonist. Liang and Donovan (1990) reported that, in cultured atrial myocytes from R-PIA treated chick embryos there was, in addition to a reduced $A_r$AR density, a conversion of receptors from high to low affinity and a reduction in $A_r$AR agonist-mediated inhibition of adenylyl cyclase. In support of these studies, Lee, et al. (1993a) demonstrated a decrease in the fraction of receptors in the high affinity state and a decrease in inhibitory G-proteins in
membranes derived from rat atria and ventricles, as well as a decrease in A,AR density in atria, but not ventricles. Dennis, et al. (1995) also demonstrated tissue-specific differences in the response to chronic A,AR agonist treatment. Guinea pigs were exposed to R-PIA for 7 days and the AV node was analyzed for agonist-induced alterations. Decreases in AV node A,AR density were accompanied by a reduced proportion of receptors in the high affinity state and decreased levels of G,α were observed. As in the study by Lee, et al. (1993a), ventricles from the same animals had decreased levels of G, and a decreased proportion of receptors in the high affinity state, with no subsequent decrease in receptor density.

Phosphorylation of occupied receptor by kinases such as G-protein coupled receptor kinases and internalization of receptors in response to chronic agonist exposure represent additional mechanisms for attenuation of receptor function. In addition to decreased A,AR density and coupling, as well as desensitization of adenylyl cyclase, Ramkumar, et al. (1991, 1993) demonstrated that A,AR was phosphorylated by the β-adrenergic receptor kinase in response to chronic agonist exposure. An increase in A,AR measured in a light vesicle preparation was also documented in R-PIA treated cells which indicated an internalization of receptors (Ramkumar, et al., 1993). Bhattacharya and Linden (1996) also reported agonist-induced internalization of A,AR in Chinese hamster ovary (CHO) cells stably expressing recombinant A,AR. Other GPCR are internalized/sequestered in response to chronic agonist exposure either via clathrin-mediated endocytosis or other internalization pathways. The most thoroughly investigated is the β-adrenergic receptor which is rapidly sequestered in response to agonist exposure and this sequestration plays a critical role in the dephosphorylation and resensitization of β2-adrenergic receptors (Yu, et al., 1993; Pippig, et al., 1995).

A study by Fernandez, et al. (1995) utilized in situ hybridization to determine whether chronic R-PIA exposure of rats altered A,AR mRNA. No
significant changes in A₁AR mRNA were documented in several brain regions, indicating that changes in receptor density produced by chronic agonist exposure are not a result of alterations in expression of A₁AR mRNA.

In response to chronic agonist exposure, A₂AAR are also altered although some of the mechanisms by which this occurs appears to differ from those of the A₁AR. In rat pheochromocytoma (PC12) cells, adenosine receptor agonist exposure inhibited the subsequent stimulation of adenylyl cyclase by A₂AAR agonists. This desensitization was not accompanied by a change in density or affinity of binding to the A₂AAR selective radioligand [³H]CGS21680 and was not mimicked by an elevation of intracellular cAMP (Chern, et al., 1993). In this study, short term exposure to agonist also did not affect A₂AAR transcript levels. Long term agonist exposure in PC12 cells produced, in addition to effects on adenylyl cyclase, a decrease in G₅α and enhanced phosphodiesterase activity (Chern, et al., 1993). Rapid desensitization of A₂AAR in response to agonist exposure was also demonstrated in CHO cells expressing the canine A₂AAR which was characterized by decreases in agonist affinity of A₂AAR and increased receptor phosphorylation (Palmer, et al., 1994). Receptors were also sequestered into a light membrane fraction which appeared to play a role in resensitization of the receptor (Palmer, et al., 1994). Longer agonist exposures resulted in downregulation of A₂AAR and upregulation of G₅α2 and G₅α3. As in the Chern, et al. (1993) study, cAMP elevation did not appear to underlie the observed changes (Palmer, et al., 1994). Conflicting results on the regulation of A₂AAR mRNA in response to chronic agonist stimulation have been reported by Saitoh, et al. (1994) and Fernandez, et al. (1995). A transient increase followed by a dramatic decrease of A₂AAR mRNA levels was induced by NECA in PC12 cells (Saitoh, et al., 1994). Forskolin was demonstrated to mimic the decrease in A₂AAR which was not due to significant reductions in message stability (Saitoh, et al., 1994). In contrast, Fernandez, et al. (1995) reported that no significant changes in A₂A adenosine receptor mRNA
measured by in situ hybridization were observed in brains from rats exposed to R-PIA for 7 days.

The more recent characterization of the $A_3$AR and development of selective agonists and antagonists has delayed the investigation of $A_3$AR regulation in response to chronic agonist exposure. However, one study utilizing an epitope tagged $A_3$AR expressed in CHO cells investigated regulation in response in chronic NECA exposure (Palmer, et al., 1995). A rapid ($t_{1/2}$ of approximately 1 minute) phosphorylation of serine or threonine residues was demonstrated which was enhanced by cotransfection with GPCR kinase 2 (GRK2) (Palmer, et al., 1995). Functional desensitization of agonist-induced inhibition of adenylyl cyclase and a 30-40% decrease in the fraction of receptors displaying high affinity agonist binding were also documented (Palmer, et al., 1995).

1.3. Statement Of Purpose

The heterogeneous expression of adenosine receptors at high density within the central nervous system (CNS) (Goodman and Snyder, 1982) suggests that adenosine plays an important role in neuromodulation. Zhang, et al. (1993) have demonstrated that endogenous adenosine exerts a tonic inhibitory modulation in the CNS through activation of $A_1$AR. The regulation of neuronal $A_1$AR is therefore of particular importance and experiments described herein were designed to elucidate the mechanisms of $A_1$AR regulation in response to chronic agonist and antagonist exposure. We have utilized primary cultures of cerebellar granule cells as a model of receptor regulation in neural tissue.
1.4. References


Chapter 2

Chronic Exposure To Adenosine Receptor Agonists And Antagonists Reciprocally Regulates The A_{1a} Adenosine Receptor-Adenylyl Cyclase System In Cerebellar Granule Cells

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2.1. Abstract

Chronic treatment with the adenosine receptor antagonist caffeine evokes an upregulation of A₁ adenosine receptors and increased coupling of the receptor to G-proteins in rat brain membranes. However, chronic agonist exposure has not been explored. Primary cultures of cerebellar granule cells were chronically exposed to A₁ adenosine receptor agonists and antagonists. Exposure to the A₁ adenosine receptor agonist, N⁶-cyclopentyladenosine resulted in: (1) a time- and concentration-dependent reduction in the density of receptors labeled by [³H]1,3-dipropyl-8-cyclopentylxanthine, (2) an enhanced ability of guanyl nucleotides to decrease the fraction of A₁ adenosine receptor sites displaying high affinity for 2-chloroadenosine, and (3) a functional uncoupling of receptors from adenylyl cyclase (EC 4.6.1.1).

The adenosine antagonists caffeine and 8-parasulfophenyltheophylline produced alterations in A₁ adenosine receptor homeostasis which were antipodal to those associated with agonist treatment. Antagonist exposure: (1) increased the density of A₁ adenosine receptors in cerebellar granule cell membranes, (2) blunted the effect of guanyl nucleotides on receptor coupling to G-proteins, and (3) increased the functional coupling of receptors to adenylyl cyclase inhibition. Forskolin treatment of cerebellar granule cells did not affect receptor density suggesting that cAMP is not involved in the regulation of A₁ adenosine receptor expression.

2.2. Introduction

Adenosine is a ubiquitous physiological regulator and neuromodulator which exerts its actions at multiple tissues, including brain, heart, kidney, vasculature, and adipocytes (Williams, 1987). Adenosine receptors are members of the G-protein coupled receptor superfamily and several subtypes have been defined on the basis of their pharmacology and/or sequence information, including A₁, A₂A, A₂B and A₃. The A₁ adenosine receptor (A₁AR) is highly expressed in brain, adipocytes and testes and may be coupled to
inhibition of adenylyl cyclase activity, activation of potassium channels, inhibition of calcium channels, and either stimulation or inhibition of phospholipase C (Linden, 1991).

Regulation of G-protein coupled receptors, G-proteins and the associated effectors provides a means for cells to adapt to chronic agonist or antagonist treatment and therefore to maintain homeostasis. The effects of chronic agonist and antagonist treatment on A₁AR have been examined in intact animals as well as cells endogenously expressing adenosine receptors from a variety of tissues. In cardiac tissues, chronic exposure to A₁AR agonists produced: (a) a decrease in the density of A₁AR (Blair and Murray, unpublished results; Shyrock, et al., 1989; Donovan, 1990; Lee, et al., 1993a; Liang and Dennis, et al., 1995), (b) a decrease in the proportion of A₁AR in the agonist high affinity state (Liang and Donovan, 1990; Lee, et al., 1993a; Dennis, et al., 1995), (c) decreased G₁α levels (Lee, et al., 1993a; Dennis, et al., 1995) and (d) blunted sensitivity to inhibition of adenylyl cyclase produced by A₁AR agonists (Liang and Donovan, 1990). Moreover, the physiological manifestations of chronic agonist exposure in the myocardium include a desensitization of negative dromotropic (Lee, et al., 1993a; Dennis, et al., 1995), inotropic (Shyrock, et al., 1989; Liang and Donovan, 1990; Lee, et al., 1993a), and chronotropic (Blair and Murray, unpublished results) effects in response to adenosine analogs. Similar results were obtained in adipocytes from animals chronically treated with A₁AR agonists; A₁AR density was decreased with a concomitant shift to the uncoupled state of the receptor, G₁α protein levels were decreased and the ability of adenosine analogs to inhibit adenylyl cyclase was blunted (Hoffman, et al., 1986; Parsons and Stiles, 1987; Green, 1987; Longabaugh, et al., 1989; Green et al., 1990; Green, et al., 1992; Olah and Stiles, 1995). The smooth muscle cell line, DDT, MF-2 has been shown to display a similar response to chronic A₁AR agonist treatment (Ramkumar, et al., 1991).
The opposing effects of chronic treatment with caffeine and other methylxanthines which act as nonselective adenosine receptor antagonists have also been investigated in a number of tissues (Reviewed by Daly, 1993). Chronic methylxanthine treatment of rats (Murray, 1982; Green and Stiles, 1986; Szot, et al., 1987; Zielke and Zielke, 1987; Ramkumar, et al., 1988; Sanders and Murray, 1989; Fastbom and Fredholm, 1990; Johannson, et al., 1993) has been shown to produce an upregulation of A<sub>1</sub>AR in various brain regions including cortex, cerebellum and hippocampus. Further analysis has shown that in addition to an increased density of receptors, the proportion of A<sub>1</sub>AR in the agonist high affinity state was increased, G<sub>1</sub>α subtype levels were increased and there was an increased ability of adenosine analogs to inhibit adenylyl cyclase (Green and Stiles, 1986; Ramkumar, et al., 1988). Similar increases in receptor density have been demonstrated in heart (Lee, et al., 1993b), adipocytes (Zhang and Wells, 1990) and DDT<sub>1</sub>, MF-2 cells (Stille and Stiles, 1991).

Adenosine receptors are heterogeneously expressed at a high density within the central nervous system (CNS) (Goodman and Snyder, 1982) suggesting that adenosine plays an important role in neuromodulation. Endogenous adenosine exerts a tonic inhibitory modulation in the CNS through activation of A<sub>1</sub>AR (Zhang, et al., 1993). The regulation of neuronal A<sub>1</sub>AR is therefore of particular importance. We have investigated the effects of chronic exposure to agonist and antagonist ligands on A<sub>1</sub>AR in cerebellar granule cells as a model of receptor regulation in neural tissue. The A<sub>1</sub>AR agonist, CPA produced a time- and concentration-dependent decrease in A<sub>1</sub>AR density without altering the affinity of the receptors for an antagonist radioligand. Chronic agonist treatment also decreased both the fraction of receptors in the agonist high affinity state (%R<sub>H</sub>) and the ability of adenosine analogs to inhibit adenylyl cyclase. Conversely, chronic antagonist treatment of cerebellar granule cell cultures resulted in an increased A<sub>1</sub>AR density, decreased ability of guanylyl nucleotides to uncouple A<sub>1</sub>AR-G-protein
complexes and an enhanced ability of adenosine analogs to produce an inhibition of adenylyl cyclase activity. The dynamic regulation of A₁AR appears to be independent of intracellular cyclic AMP levels inasmuch as forskolin did not affect A₁AR expression.

2.3. Materials And Methods

2.3.1. Materials

[^3]H]1,3-dipropyl-8-cyclopentylxanthine ([^3]H]DPCPX; 109 Ci/mmol), [^3]H]adenine (26.9 Ci/mmol), [[^14]C]cyclic AMP ([[^14]C]cAMP; 52.3 mCi/mmol) were purchased from NEN-Dupont (Boston, MA). Trypsin, DNase, trypsin inhibitor, glutamine, polylysine, gentamycin, fetal bovine serum, cytosine arabinoside (araC), caffeine, guanosine-5′-(β-imido)triphosphate (Gpp(NH)p), 2-chloroadenosine (2-CADO), glucose, forskolin and neutral alumina were from Sigma Chemical Company (St. Louis, MO). N⁶-cyclopentyladenosine (CPA), 8-parasulfophenyltheophylline (8-pSPT) and 4-[(3-butoxy-4-methoxypheny)methyl]-2-imidazolidinone (RO20-1724) were from Research Biochemicals, Inc. (Wayland, MA). Adenosine deaminase was from Boehringer Mannheim (Mannheim, Germany). Basal Eagle’s media was from Gibco (Gaithersburg, MD). Cytoscint was from ICN Radiochemicals (Irvine, CA). Dowex A6 50W-X4 was from Biorad (Richmond, CA).

2.3.2. Preparation Of Cerebellar Granule Neurons

Rat cerebellar granule neurons were prepared from 8-day old Sprague Dawley rat pups as described previously (Novelli, et al., 1988). The protocol used was approved by the Institutional Animal Care and Use Committee of Oregon State University and all animals used in these studies were housed in accordance with "Principles for Use of Animals and Guide for the Care and Use of Laboratory Animals", NIH Pub. No., 85-23. Briefly, cerebella were dissected and dissociated with trypsin and DNase, followed by addition of
trypsin inhibitor to limit digestion. Cells were physically dissociated and suspended in depolarizing media, containing basal Eagle's media, 25 mM KCl, 2 mM glutamine, 10% fetal bovine serum and 100 μg/ml gentamycin. Cells were then plated on polylysine coated (10 μg/ml) 100 mm culture plates (Falcon) or 6-well, 35 mm plates (for adenylyl cyclase assays). AraC (10 μM) was added to the culture medium after 24 hours to limit replication of non-neuronal cells. Cells were maintained at 37° C in an atmosphere of 5% CO₂/95% air and 95% humidity. Caffeine, 8-pSPT and CPA stocks (100X) were prepared in distilled water, sterile filtered and added at the indicated concentrations and times.

2.3.3. Cerebellar Granule Cell Membrane Preparation

Cells were rinsed three times with 10 ml of phosphate buffered saline to remove media and drugs prior to membrane preparation. Cells were then scraped into 50 mM Tris, pH 7.5 at 4° C and homogenized with 10-12 strokes in a Dounce homogenizer. The homogenate was centrifuged at 44,000 x g in a Beckman JA-20 rotor at 4° C for 10 minutes. The pellet was resuspended in 50 volumes of cold 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 using a Dounce homogenizer. This homogenate was centrifuged at 44,000 x g for 10 minutes at 4° C, washed twice in cold 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 and centrifuged as above. The resulting pellet was resuspended in 20 volumes of 22° C 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 with 2.5 U/ml adenosine deaminase and incubated 120 minutes at 22° C in order to remove endogenous adenosine. Treated membranes were centrifuged as above and resuspended in 22° C 50 mM Tris, 1 mM EDTA, pH 7.7, 0.5 U/ml adenosine deaminase for binding assays.

2.3.4. Binding Of [³H]DPCPX in Cerebellar Granule Cells.

The specific binding of the A₁-selective ligand [³H]DPCPX to granule cell membranes was determined using a previously described rapid filtration
assay (Blair, et al., 1989). Cerebellar granule cell membrane preparations (70-100 µg of protein) in a final volume of 250 µl were incubated for 90 minutes at 22° C with [3H]DPCPX in the presence or absence of competing compound. The equilibrium binding reactions were terminated by vacuum filtration using a Brandel cell harvester (M-48R) and Whatman GF/C filters saturated with 0.5% polyethylenimine. Each filter was washed four times with 4 ml cold 50 mM Tris-HCl, pH 7.7. Filter-bound radioactivity was determined by liquid scintillation counting (Beckman Model LS 6000SC). Nonspecific binding was defined in the presence of 100 µM CPA. In saturation binding experiments, [3H]DPCPX was used at 8-12 concentrations, ranging from 0.05 to 5.0 nM. All other experiments included [3H]DPCPX at approximately 1.0-1.5 nM.

2.3.5. Adenylyl Cyclase Assay

Adenylyl cyclase activity in cerebellar granule cells was assayed as described by Salomon (1979). Cells were rinsed with serum-free media three times. Serum-free media containing [3H]adenine (1.2 µCi/ml) was added to the cells and incubated for 2 hours at 37°C in order to allow uptake of [3H]adenine into the cells. [3H]adenine containing media was aspirated and cells were rinsed three times with serum-free media. Serum free media containing 50 µM of the phosphodiesterase inhibitor, RO20-1724, 0.5 µM forskolin or vehicle (ethanol), and varying concentrations of CPA was added to cells. After a 20 minute incubation at 37°C, the reactions were terminated by aspiration followed by addition of 300 µl stop solution containing 1.3 mM cAMP and 2% SDS (w/v). To each plate, 50 µl [14C]cAMP stock was added to control for recovery of cAMP, followed by 750 µl water and 100 µl perchloric acid to lyse labeled cells. The contents of the plate were then transferred to Eppendorf tubes and 100 µl 12 M KOH was added to neutralize the perchloric acid. This solution was centrifuged 15 minutes at 9000 rpm at 4°C and the supernatant was subjected to sequential chromatography over Dowex A6
50W-X4 and neutral alumina columns and the eluate was counted after addition of 10 ml scintillation cocktail.

2.3.6 Protein Determination
Membrane protein content was assayed by the method of Lowry, et al. (1951) after solubilization of the samples in 0.5 N NaOH. Bovine serum albumin was used as the standard.

2.3.7. Data Analysis
Equilibrium saturation binding and adenylyl cyclase inhibition data were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). Saturation isotherms were analyzed by fitting a hyperbolic equation to the \(^{3}H\)DPCPX specific binding data. Adenylyl cyclase inhibition data were fit with a logistic equation:

\[
Y = \frac{\text{max.} - \text{min.}}{1 + (\text{EC}_{50}/X)^n}
\]

where \(Y\) is the response, \(X\) is CPA concentration, max and min the maximum and minimum plateaus of the concentration response curve, \(\text{EC}_{50}\) is the concentration of CPA producing 50% inhibition and \(n\) the slope factor.

Agonist binding data were fit assuming two non-interacting classes of sites. In accordance with the ternary complex model of DeLean, et al. (1980) these two classes of sites represent free receptor which binds agonist with low affinity, and receptor coupled to G-protein which displays high affinity agonist binding. The ternary complex model accounts for Gpp(NH)p-induced reduction in agonist binding as a consequence of the uncoupling of receptor-G protein complexes with attendant decrements in the fraction of high affinity sites. Agonist binding data were therefore fit using the equation below:

\[
[Y] = \frac{\%R_H}{[1+10^{\beta L}]} + \frac{100-\%R_H}{[1+10^{\beta L}]}
\]
where \([Y]\) is the amount of \([^3\text{H}]\)DPCPX specifically bound, \(\%R_h\) the fraction of high affinity sites, \(L\) the ligand concentration, and \(A\) and \(B\) the \(\log(\text{IC}_{50})\) values for high and low agonist affinity constants, respectively. These equilibrium competition data were fit by nonlinear least squares regression using the method of global analysis in Origin (MicroCal Software, Inc.). Combined data from multiple experiments were fit using shared affinity constants and unique fractions for high affinity binding (\(\%R_h\)). The two-site model significantly improved the fit compared to that obtained from a one-site model for all 2-CADO titration curves. IC\(_{50}\) values for high- and low-affinity agonist binding sites were converted to corresponding \(K_h\) and \(K_l\) values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

2.4. Results

2.4.1. Agonist-Induced Alterations in \(\text{A}_1\) Adenosine Receptors

Exposure of cerebellar granule cell cultures to the \(\text{A}_1\) selective agonist, CPA (10 \(\mu\)M) produced a time-dependent decrease in binding of the \(\text{A}_1\) selective antagonist radioligand, \([^3\text{H}]\)DPCPX as shown in Figure 1.A. Binding of \([^3\text{H}]\)DPCPX in CPA treated membranes was reduced by 6, 38, 36, and 32\% following 1, 2, 3, and 5 days of exposure, respectively. To characterize the concentration-response relationship for CPA-induced decrease in \([^3\text{H}]\)DPCPX binding, granule cell cultures were exposed for 3 days to a range of CPA concentrations and analyzed for \([^3\text{H}]\)DPCPX binding. The concentration-dependent decrease in binding represented in Figure 1.B. revealed that the concentration of CPA required to produce a half maximal reduction in \([^3\text{H}]\)DPCPX binding was 4.3 \pm 1.8 nM. A 3 day exposure to 10 \(\mu\)M CPA produced a significant reduction in \([^3\text{H}]\)DPCPX binding and was used to further investigate the \(\text{A}_1\)AR downregulation in subsequent experiments. In order to determine whether alterations in \(\text{A}_1\)AR binding were due to a change
Fig. 1. Time- and concentration- dependence of CPA-induced
downregulation of A1AR. (A) [$^3$H]DPCPX binding in cerebellar granule cell
membranes as a function of exposure time. 10 μM CPA or vehicle was added
to cerebellar granule cells on day 5 in culture. Membranes were collected as
per methods section after a 1, 2, 3, or 5 day exposure and [$^3$H]DPCPX binding
was assessed. Nonspecific binding was defined by the addition of 100 μM
CPA. The data shown are the means of triplicates from three experiments.
Specific binding is represented as the percent of control on each day. (B)
Effect of CPA concentration on [$^3$H]DPCPX binding in cerebellar granule cells.
A range (10^-4 to 10^-8 M) of CPA concentrations and vehicle only were added to
granule cells on day 5 in culture. Membranes were collected after a 3 day
exposure and assayed for [$^3$H]DPCPX binding. The data shown are means of
triplicates from two experiments. *[$^3$H]DPCPX binding in CPA treated cells
determined to be significantly different from control (p<0.05).
Fig. 2. [³H]DPCPX saturation binding in vehicle and CPA treated cerebellar granule cells. Granule cells were exposed to vehicle or 10 μM CPA for 3 days. (A) The squares represent binding in vehicle treated cells and the triangles represent binding in CPA treated cells. The data shown are derived from a representative experiment which was repeated twice. The fit shown was obtained using nonlinear regression analysis, which yielded a $K_D$ of 0.39 ± 0.02 nM and a $B_{max}$ of 102.5 ± 1.2 fmol/mg protein for vehicle treated cells and a $K_D$ of 0.29 ± 0.06 nM and $B_{max}$ of 56.4 ± 3.1 fmol/mg protein for CPA treated cell membranes. Closed symbols indicate specific binding and open symbols nonspecific binding. (B) Scatchard replot of these saturation data.
in receptor number or \(^3\text{H}\)DPCPX affinity, saturation analysis of membranes from cerebellar granule cultures incubated for 3 days in the presence or absence of 10 \( \mu \text{M} \) CPA were performed. Representative saturation binding curves for CPA and vehicle treated cells are shown in Figure 2A and the Scatchard replot of these data in Figure 2B. CPA exposure effected a significant decrease in the density of A\(_r\)AR as labeled by \(^3\text{H}\)DPCPX. The control \(^3\text{H}\)DPCPX \(B_{\text{max}}\) value of 102.5 ± 1.2 fmol/mg protein was reduced to 56.4 ± 3.1 fmol/mg protein in CPA exposed membranes. The \(K_D\) values for \(^3\text{H}\)DPCPX binding to the A\(_r\)AR in CPA exposed cells were unchanged from those of vehicle controls (Control \(K_D = 0.39 \pm 0.2 \text{ nM} \); CPA exposed \(K_D = 0.29 \pm 0.06 \text{ nM} \)). The correspondence of the \(K_D\) values in control and treated membranes indicates that the washing and membrane preparation procedures employed effectively eliminated residual CPA from the binding assay.

To further analyze the effect of chronic agonist treatment on ligand interaction with A\(_r\)AR, agonist competition of \(^3\text{H}\)DPCPX binding was used to ascertain the effects of CPA treatment on the functional coupling of A\(_r\)AR with G-proteins. These data derived from equilibrium competition experiments depicted in Figure 3 are best described by a model for ligand-receptor interaction at two independent classes of sites. Global analysis of the multiple data sets depicted in Figure 3 yielded \(K_H\) and \(K_L\) values for 2-CADO of 11.8 ± 0.3 nM and 1.56 ± 0.28 \(\mu\text{M}\), respectively. Treatment of cerebellar granule cells with CPA decreased the fraction (%\(R_H\)) of A\(_r\)AR in the high-affinity state. As indicated in Table 1, CPA exposure effected a 30% decrease in the fraction of high-affinity sites \((p \leq 0.05)\), while the Gpp(NH)p-induced reduction in the fraction of high-affinity sites was significantly \((p<0.05)\) augmented. These data indicated that a 3 day exposure of intact neurons to CPA decreased the A\(_r\)AR-G-protein coupling efficiency.
Fig. 3. Competition of $[^3]H$DPCPX binding by 2-CADO in granule cell membranes from vehicle, CPA and 8-pSPT treated cells in the absence and presence of 300 μM Gpp(NH)p. Granule cell membranes were incubated with 1.5 nM $[^3]H$DPCPX and increasing concentrations of 2-CADO. The 2-CADO titrations were simultaneously fit using shared high- and low- affinity constants and unique fraction (%$R_h$) of high affinity agonist sites. The resultant parameter estimates were used to generate depicted curves from expansion of the two-site equation given in the methods section. Control values are indicated by circles, CPA by squares, and 8-pSPT by triangles. Closed symbols and solid lines represent membranes incubated in the absence of Gpp(NH)p and open symbols and dotted lines represent membranes incubated in the presence of Gpp(NH)p.
TABLE 1. Influence of 3-day exposure to CPA or 8-pSPT on the fraction of high-affinity sites (%RH) in 2-CADO titration of A1AR in membranes derived from cerebellar granule cells

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>%RH -Gpp(NH)p</th>
<th>%RH + 300 μM Gpp(NH)p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.7 (46-58)</td>
<td>30.5 (25-36)a</td>
</tr>
<tr>
<td>CPA</td>
<td>36.4 (31-42)b</td>
<td>8.1 (1-15)a,b</td>
</tr>
<tr>
<td>8-pSPT</td>
<td>48.2 (44-53)</td>
<td>39.2 (34-44)</td>
</tr>
</tbody>
</table>

The values represent the fraction (%RH) of total [3H]DPCPX binding sites displaying high affinity for the agonist 2-CADO. The 2-CADO titration curves shown in Fig. 3 were fit to a two-site model as described in Materials and Methods. 2-CADO titrations in control, CPA- and 8-pSPT-exposed cells were simultaneously fit using shared high- and low-affinity constants and unique fractions (%RH) for high-affinity agonist sites. The fitted values for the 2-CADO high- and low- constants (K_H and K_L) were, respectively, 11.8 ± 0.3 nM and 1.56 ± 0.28 μM. The %RH values in this table were derived from analyses of data pooled from several experiments. The 95% confidence intervals are indicated in parentheses. a Significantly different from corresponding value in absence of Gpp(NH)p. b Significantly different from corresponding control value.

2.4.2. Effect Of Chronic Agonist Exposure On Adenosine Analog-Induced Inhibition Of Adenylyl Cyclase

The functional consequences of CPA-induced downregulation of A1AR were assessed by determining the ability of CPA to inhibit adenylyl cyclase in control and CPA exposed cells. These assays provide a measure of A1AR coupling to adenylyl cyclase through G-proteins. In vehicle treated cells, increasing concentrations of CPA produced a dose-dependent decrease in adenylyl cyclase activity as depicted in Figure 4. Adenylyl cyclase activity was maximally inhibited by 45.5 ± 3.1% with an IC_50 of 39.4 ± 1.7 nM in vehicle treated cells. In contrast, a 3 day exposure to 10 μM CPA resulted in a blunted inhibition of adenylyl cyclase activity with the maximal inhibition being significantly reduced to 18.1 ± 7.6% (p<0.01).
Fig. 4. Effect of CPA exposure on the CPA-induced inhibition of adenylyl cyclase activity. Cerebellar granule cells were treated with vehicle, 10 \( \mu \)M CPA for 3 days and assayed for adenylyl cyclase activity. Data points represent the mean values of triplicate determinations from three experiments. Decreased sensitivity of CPA-induced inhibition of adenylyl cyclase activity as a result of 3 day CPA exposure. The maximal inhibition in vehicle treated cells was 45.5% with an IC\textsubscript{50} of 39.4 nM whereas the maximal inhibition of cyclase in CPA treated cells was 18.1% with an IC\textsubscript{50} of 83.1 nM.

2.4.3. Effect Of Antagonist Exposure On A\textsubscript{1} Adenosine Receptor Binding In Cerebellar Granule Cells

In contrast to the downregulation of A\textsubscript{1}AR in response to chronic agonist exposure, chronic antagonist exposure resulted in an increase in \[^{3}H\]DPCPX binding. As shown in Figure 5A, the nonselective adenosine receptor antagonist, caffeine (100 \( \mu \)M) produced a time-dependent increase in \[^{3}H\]DPCPX binding. A 3 day treatment period resulted in the maximal upregulation of binding and was therefore used in subsequent experiments. To further characterize the increase in \[^{3}H\]DPCPX binding resulting from caffeine exposure, cerebellar granule cell cultures were exposed to various concentrations of caffeine and \[^{3}H\]DPCPX binding was determined as shown in Figure 5B. Caffeine produced a concentration-dependent increase in \[^{3}H\]DPCPX binding with maximal increments occurring at a caffeine
concentration of 100 μM. The concentration of caffeine required to produce the half maximal increase was $2.8 \pm 1.9 \text{ μM}$. Equilibrium saturation analysis indicated that a 3 day exposure to 100 μM caffeine resulted in a 16% increase in the $[^{3}\text{H}]$DPCPX $B_{\text{max}}$ value with no significant change in receptor affinity (Figure 6). Vehicle treated cells had a $B_{\text{max}}$ value of $85.0 \pm 5.2$ fmol/mg protein and $K_{D}$ of $0.45 \pm 0.09$ nM whereas the $B_{\text{max}}$ for caffeine treated cells was $98.5 \pm 5.4$ fmol/mg protein with a $K_{D}$ of $0.39 \pm 0.07$ nM.

In addition to its actions as an antagonist at adenosine receptors, caffeine is an inhibitor of phosphodiesterase. It is therefore possible that the phosphodiesterase inhibitor activity could contribute to the caffeine-induced upregulation of $A_{1}$AR. In order to isolate the effect of receptor antagonism on $A_{1}$AR upregulation, the influence of 8-pSPT on $[^{3}\text{H}]$DPCPX interaction with $A_{1}$AR was assessed. The adenosine receptor antagonist 8-pSPT is impermeant. This restriction to the extracellular compartment excludes an interaction of 8-pSPT with phosphodiesterases and renders this compound a selective inhibitor of adenosine receptors. Figure 5C depicts the concentration-response curve for 8-pSPT-induced upregulation of $[^{3}\text{H}]$DPCPX binding sites in cerebellar granule cell membranes. At 100 μM, 8-pSPT elicited a maximal 38% increase in $[^{3}\text{H}]$DPCPX binding. The half maximal effect was produced by $8.6 \pm 1.4 \text{ μM}$ 8-pSPT. The potencies for caffeine and 8-pSPT to evoke an upregulation of $A_{1}$AR are in reasonable agreement with their affinities for $A_{1}$AR which are respectively 100 and 14 μM (data not shown). Equilibrium saturation analyses of membranes from cells treated with vehicle or 100 μM 8-pSPT are represented in Figure 7. Treatment with 8-pSPT produced a 47% increase in the $[^{3}\text{H}]$DPCPX $B_{\text{max}}$ value ($p < 0.01$) while the $K_{D}$ remained unchanged. The $B_{\text{max}}$ of vehicle treated cells was $77.1 \pm 3.8$ fmol/mg protein with a $K_{D}$ of $0.35 \pm 0.06$ nM and the $B_{\text{max}}$ of 8-pSPT treated cells was $113.6 \pm 3.3$ with a $K_{D}$ of $0.36 \pm 0.04$ nM.
Fig. 5. Time- and concentration- dependence of caffeine and 8-pSPT exposure on [³H]DPCPX binding. (A) Effect of length of caffeine treatment on [³H]DPCPX binding. Caffeine (100μM) or vehicle was added to cells on day 5 in culture and membranes were collected for [³H]DPCPX binding after the
indicated exposure times. The data shown here are the mean values of duplicates from three experiments. (B) Effect of caffeine concentration of \([^3H]DPCPX\) binding in cerebellar granule cells. Caffeine (10\(^{-4}\) to 10\(^{-8}\) M) or vehicle was added to granule cells for 3 days and \([^3H]DPCPX\) binding was assessed. Data are the mean values of duplicates from three experiments. (C) Effect of 8-pSPT concentration on \([^3H]DPCPX\) binding in cerebellar granule cell membranes. Vehicle or 8-pSPT (10\(^{-4}\) to 10\(^{-8}\) M) was added to cultures on day 5. Cell membranes were assayed for \([^3H]DPCPX\) binding after a 3-day exposure. Data are mean values of duplicates from two experiments. \([^3H]DPCPX\) binding in caffeine- or 8-pSPT-treated cells was determined to be significantly different from control (*p < 0.05).

**Fig. 6.** \([^3H]DPCPX\) saturation binding in vehicle and caffeine treated cerebellar granule cells. Granule cells were exposed to vehicle or 100 µM caffeine for 3 days and membranes prepared as described in the methods. \([^3H]DPCPX\) (0.05 to 5.0 nM) was incubated with membranes and nonspecific binding was defined by the addition of 100 µM CPA. (A) The squares represent binding in vehicle treated cells and the triangles represent binding in caffeine treated cells. The data shown here are the means of duplicates from an experiment that is representative of three performed. The fit shown yielded a \(K_D\) of 0.45 ± 0.09 nM and a \(B_{max}\) of 85.0 ± 5.2 fmol/mg protein for vehicle treated cells and a \(K_D\) of 0.39 ± 0.07 nM and \(B_{max}\) of 98.5 ± 5.4 fmol/mg protein for caffeine treated cell membranes. Closed symbols indicate specific binding and open symbols indicate nonspecific binding. (B) Scatchard replot of these saturation data.
Fig. 7. \[^{3}H\]DPCPX saturation binding in vehicle and 8-pSPT treated cerebellar granule cells. Granule cells were exposed to vehicle or 100 μM 8-pSPT for 3 days and membranes were prepared as described in the methods. (A) The squares represent binding in vehicle treated cells and the triangles represent binding in 8-pSPT treated cells. The data shown here are the means of duplicates from an experiment that is representative of the three performed. The fit shown yielded a $K_D$ of 0.35 ± 0.06 nM and a $B_{max}$ of 77.1 ± 3.8 fmol/mg protein for vehicle treated cells and a $K_D$ of 0.36 ± 0.04 nM and $B_{max}$ of 113.6 ± 3.3 fmol/mg protein for 8-pSPT treated cell membranes. Closed symbols indicate specific binding and open symbols indicate nonspecific binding. (B) Scatchard replot of these saturation data.

In contrast to the results from CPA exposed neurons, treatment of cerebellar granule cells with the antagonist 8-pSPT resulted in a blunted ability of Gpp(NH)p to uncouple A₁AR-G-protein complexes inasmuch as Gpp(NH)p reduced the fraction of high-affinity sites by 40% in control membranes and only 19% in 8-pSPT exposed membranes.

2.4.4. Effect of chronic antagonist exposure on adenosine analog induced inhibition of adenylyl cyclase

A₁AR coupling to adenylyl cyclase was assessed in vehicle and 8-pSPT treated cells in Figure 8. In vehicle treated cells, CPA produced a concentration-dependent decrease in adenylyl cyclase activity with an $IC_{50}$ of 31.4 ± 1.2 nM and maximal inhibition of 49.5 ± 1.9%. Exposure of cerebellar
granule cells to 100 μM 8-pSPT for 3 days resulted in a shift in the IC_{50} to 14.8 ± 1.2 nM of the CPA-induced decrease in adenylyl cyclase activity that did not reach statistical significance. The maximum level of CPA inhibition of adenylyl cyclase activity in 8-pSPT treated cells was not significantly different (47.8 ± 2.5%) from control.

Fig. 8. Effect of 8-pSPT exposure on the CPA-induced inhibition of adenylyl cyclase activity. Cerebellar granule cells were treated with vehicle or 100 μM 8-pSPT for 3 days and assayed for adenylyl cyclase activity. Data points represent the mean values of triplicate determinations from three experiments. The maximal inhibition in vehicle treated cells was 51.8% with an IC_{50} of 31.4 nM whereas the maximal inhibition of cyclase in 8-pSPT treated cells was 56.2% with an IC_{50} of 14.8 nM.

2.4.5. Effect of forskolin exposure on [3H]DPCPX binding
To determine the potential role of cAMP levels on A_{1}AR regulation, the influence of forskolin on [3H]DPCPX binding to cerebellar granule cell membranes was determined. Cultures exposed to 10 μM forskolin for varying lengths of time showed no significant changes in [3H]DPCPX binding (Figure 9). Moreover a 3 day exposure to a range of forskolin concentrations failed to
affect $[^3]H$DPCPX $K_d$ or $B_{max}$ values (data not shown). These results suggest that adenyl cyclase activity and/or intracellular cAMP levels do not play a role in the regulation of $A_r$AR expression.

**Fig. 9.** Effect of forskolin exposure on $[^3]H$DPCPX binding. 10 μM forskolin or vehicle was added to cells on day 5 in culture and membranes were collected for $[^3]H$DPCPX binding at the indicated exposure times. 100 μM CPA was used to define nonspecific binding. The data shown here are expressed as percent of $[^3]H$DPCPX bound to vehicle treated membranes and are the means of triplicates from two experiments.

**2.5. Discussion**

The results of these studies demonstrate that neuronal $A_r$AR are dynamically regulated in response to chronic exposure to agonist and antagonist ligands. The response of cerebellar granule cells to prolonged exposure to the $A_r$AR selective agonist CPA was manifold. $A_r$AR density was decreased in a time- and concentration-dependent manner. The $[^3]H$DPCPX $K_d$ values were identical in control and treated membranes suggesting that the downregulation was not associated with alterations in post-translational
modifications of receptor structure. Equilibrium agonist competition of 
antagonist binding was best described by a model for ligand interaction at two 
independent classes of sites. In membranes from cultures exposed to CPA, 
a lower fraction of A₁AR recognized 2-CADO with high affinity. Moreover, 
Gpp(NH)p had a significantly greater effect on agonist binding in CPA- as 
compared to vehicle-treated cells. This effect on agonist titration curves 
manifested as an augmented shift in the fraction of agonist binding sites from 
high to the low affinity state. Gpp(NH)p evoked a 41% decrease in %Rₜ in 
control membranes and a 78% decrease in CPA exposed membranes. These 
results suggest that A₁AR in CPA treated cells are not as tightly coupled to G- 
proteins as those in vehicle treated cells.

Cells chronically exposed to CPA were also subsensitive to A₁AR 
agonist-elicited adenylyl cyclase inhibition when compared to vehicle treated 
cells. This decreased ability of CPA to inhibit adenylyl cyclase appears to 
result from the decreased density of A₁AR, as well as the decreased efficiency 
of receptor-G-protein coupling.

Although downregulation of neuronal A₁AR has not been reported 
previously, the downregulation of A₁AR observed in cerebellar granule cells is 
consonant with that observed with A₁AR signaling in a variety of other tissues 
including heart, adipocytes, and DDT, MF-2 smooth muscle cells. Previous 
reports using embryonic chick heart exposed in ovo to the A₁AR selective 
adenosine receptor agonist, (+)-N⁶-(R-phenylisopropyl-adenosine) (R-PIA) for 
44 hours resulted in decreased density of A₁AR as observed herein. Also in 
chick heart, the efficacy of R-PIA to produce a decrease in contractile tension 
was attenuated in hearts chronically treated with R-PIA demonstrating a 
functional consequence of the A₁AR downregulation (Shyrock, et al., 1989). 
More recently Dennis, et al. (1995) characterized a desensitization of the 
guinea pig AV node resulting from a 7 day exposure to R-PIA which produced, 
in addition to a decrease in A₁AR density, a reduced proportion of receptors in 
the high affinity state (%Rₜ). This downregulation in AV nodal tissue was
accompanied by a decrease in the levels of the inhibitory G-protein coupled to $A_1$ adenosine receptors, $G_{\alpha}$. The loss of high affinity binding sites in desensitized AV nodal tissue may, in part, be due to the decrease in $G_{\alpha}$. Similar results have been reported in R-PIA treated chick cardiac myocytes (Liang and Donovan, 1990) and in rat atria (Lee, et al., 1993a). In the latter study, atria of rats infused with R-PIA for 7 days exhibited a decrease in $A_1$AR density, a decrease in proportion of receptors in the high affinity state and a decrease in $G_{\alpha}$. Interestingly, ventricles from the same animals had decreased levels of $G_{\alpha}$ and a decreased proportion of receptors in the high affinity state, with no subsequent decrease in receptor density. Considered together, these results suggest that tissue-specific differences in the response to chronic $A_1$AR agonist treatment may exist.

Adipocytes have provided another model for the investigation of $A_1$AR regulation in response to chronic agonist treatment. In rat adipocytes, a number of studies have demonstrated desensitization following chronic exposure to R-PIA (Hoffman, et al., 1986; Parsons and Stiles, 1987; Green, 1987; Longabaugh, et al., 1989; Green, et al., 1990; Green, et al., 1992; and reviewed in Olah and Stiles, 1995). Similar to the results presented herein with cerebellar granule cells, Parsons and Stiles (1987) demonstrated that chronic R-PIA exposure produced an attenuation of the agonist-induced inhibition of forskolin-stimulated adenylyl cyclase. Treatment with R-PIA also produced a decrease in the fraction of receptors in the high affinity binding state (Parsons and Stiles, 1987). In agreement with this latter report, CPA exposure of cerebellar granule cells was associated with a reduction in the fraction of receptors in the agonist high-affinity state representing a decreased efficiency of receptor-G-protein coupling.

Additional evidence of a functional uncoupling and downregulation of $A_1$AR in adipocytes was provided by Longabaugh, et al. (1989). These authors observed parallel decrements in $G_{\alpha}1$ and $G_{\alpha}2$ levels in response to a 6 day in vivo treatment with R-PIA (Longabaugh, et al., 1989). Green, et al.
(1990) also reported a down-regulation of A<sub>1</sub>AR and an attenuation of G-protein levels, specifically G<sub>13</sub>1 and G<sub>13</sub>3, in adipocytes chronically exposed to R-PIA. These changes in G-protein levels with chronic agonist treatment may contribute to the decreased high-affinity binding and blunted ability of adenosine analogs to inhibit adenylyl cyclase. Similarly Hoffman, et al. (1986) reported a decreased ability of R-PIA to inhibit cAMP formation in adipocytes from R-PIA infused rats as measured by inhibition of isoproterenol-stimulated adenylyl cyclase. This decreased ability was characterized by a dramatic shift of the dose-response curve to the right whereas the maximum effect remained the same (Hoffman, et al., 1986). In DDT, MF-2 cells chronically treated with R-PIA, inhibition of isoproterenol-stimulated adenylyl cyclase activity produced both an attenuation of the maximal inhibition produced by R-PIA and a 20-fold increase in the IC<sub>50</sub> (Ramkumar, et al., 1991).

The role of intracellular cAMP in the regulation of A<sub>1</sub>AR expression was explored as a possible underlying mechanism for the CPA-induced downregulation. Other G<sub>13</sub>-coupled inhibitory receptors such as the α2-adrenergic (Sakaue and Hoffman, 1991), D<sub>2L</sub> dopamine (Johansson and Westlind-Danielsson, 1994) and somatostatin (Greenwood, et al., 1994) receptors appear to be transcriptionally regulated by cAMP; elevation of cAMP is associated with increased expression of these receptors. Thus, a parsimonious hypothesis to account for CPA-induced downregulation of A<sub>1</sub>AR would be that the CPA inhibition of adenylyl cyclase would reduce intracellular cAMP levels which in turn may reduce a stimulus for the expression of A<sub>1</sub>AR. The effects of CPA exposure on A<sub>1</sub>AR density do not appear to involve cAMP inasmuch as forskolin treatment of cultures did not affect A<sub>1</sub>AR expression.

Agonist-induced phosphorylation of occupied receptor by kinases such as G-protein coupled receptor kinases represent an additional mechanism for modification of receptor function. Indeed phosphorylation of the A<sub>1</sub>AR by the β-adrenergic receptor kinase has been shown to effect an uncoupling of the A<sub>1</sub>AR from G-proteins (Ramkumar, et al., 1993). This uncoupling from G-
proteins therefore represents an important mechanism underlying
desensitization. Receptor phosphorylation may also contribute to
sequestration and ultimate degradation of receptors inasmuch as β-adrenergic
receptor kinase I mediated phosphorylation of the β2-adrenergic receptor
facilitates sequestration by promoting the interaction of arrestin with the
receptor (Ferguson, et al., 1996). Thus, the observed downregulation of A1AR
could result from CPA-enhanced internalization and degradation of the
receptor. In support of the role of sequestration in the process of
downregulation, we have observed that neurons chronically exposed to CPA
have an increased fraction of sequestered receptors (unpublished results).

We have also assessed the response of A1AR to chronic antagonist
exposure in cerebellar granule neurons. Exposure to either caffeine or 8-
pSPT resulted in an increased A1AR receptor density whereas the affinity of
the receptors for the antagonist radioligand remained unchanged. Exposure
to the antagonist 8-pSPT affected A1AR coupling to G-proteins as evidenced
by a blunted sensitivity of 2-CADO titration curves to Gpp(NH)p in 8-pSPT
treated membranes. Moreover, a three day exposure to 100 µM 8-pSPT
resulted in a sensitization of the CPA-induced inhibition of adenylyl cyclase,
represented by a leftward shift of the dose-response curve. This sensitization
of adenylyl cyclase following 8-pSPT exposure is presumably a result of the
increase in A1AR density and enhanced receptor-G-protein coupling.

The effects of chronic antagonist treatment in cerebellar granule cells is
similar to the upregulation of A1AR demonstrated in a variety of tissues
isolated from rats chronically exposed to chronic caffeine, theophylline or
related methylxanthines (Murray, 1982; Green and Stiles, 1986; Szot, et al.,
1987; Zielke and Zielke, 1987; Ramkumar, et al., 1988, Sanders and Murray,
1989; Fastbom and Fredholm, 1990; Zhang and Wells, 1990; Johannson, et
al., 1993; Lee, et al., 1993; and reviewed in Daly, 1993) as well as in the
smooth muscle cell line, DDT, MF-2 (Stille and Stiles, 1991). In rat, chronic
theophylline treatment produced an upregulation of A1AR binding in cortical
(Murray, 1982; Szot, et al., 1987; Sanders and Murray, 1989) and cerebellar (Szot, et al., 1987) membranes. In rats, exposed orally to caffeine, A<sub>1</sub>AR binding in the CA3 subfield of the hippocampus increased, as demonstrated by Johansson, et al. (1993) using autoradiography with the agonist radioligand N<sup>6</sup>-cyclohexyl-[<sup>3</sup>H]adenosine ([<sup>3</sup>H]CHA). In addition to the changes in receptor density in response to chronic antagonist treatment, changes in the proportion of receptors in the high affinity state, levels of G<sub>a</sub>, and the maximal inhibition of adenylyl cyclase produced by adenosine analogs have been reported in rat cerebral cortical membranes (Ramkumar, et al., 1988; Green and Stiles, 1986). These results correspond to the present results in cerebellar granule cell membranes. The observed increases in receptor density could be a result of increased transcription of the A<sub>1</sub>AR gene and/or increased mRNA stability. Alternatively, the antagonist induced upregulation of A<sub>1</sub>AR may result from relief of the tonic inhibitory tone exerted by adenosine in the medium. Attendant to this inhibitory tone would be a basal level of downregulation of A<sub>1</sub>AR which may be reversed by antagonist occupancy of the A<sub>1</sub>AR.

In summary, chronic exposure of intact neurons to A<sub>1</sub>AR agonists leads to a decreased density of A<sub>1</sub>AR and reduced efficiency of A<sub>1</sub>AR coupling to G-proteins. These adaptive responses, in turn, subserve the decreased responsiveness of neurons to agonist-induced inhibition of adenylyl cyclase. Conversely, we have shown that chronic treatment of neuronal cultures with antagonists to A<sub>1</sub>AR results in an increase in receptor density and altered receptor G-protein coupling. This adaptive upregulation underlies the increased sensitivity of the cells to inhibition of adenylyl cyclase in response to A<sub>1</sub>AR agonists. The fundamental role of adenosine as an inhibitory neuromodulator and endogenous anticonvulsant in the central nervous system renders the understanding of the mechanisms underlying A<sub>1</sub>AR regulation of considerable significance.
2.6. Acknowledgments

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2.7. References


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Cyclopentyladenosine-Induced Homologous Downregulation Of A₁ Adenosine Receptors (A₁AR) In Intact Neurons Is Accompanied By Sequestration But Not A Reduction In A₁AR mRNA Expression Or G-Protein α-Subunit Content

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3.1. Abstract

Exposure of cerebellar granule cells to the A1 adenosine receptor-selective agonist, cyclopentyladenosine produced a decrease in A1 adenosine receptor density and G-protein coupling which corresponded to a blunted agonist-induced inhibition of adenylyl cyclase (Hettinger-Smith, et al., 1996). We have now determined that A1 adenosine receptor-mediated inhibition of adenylyl cyclase was desensitized in a homologous manner in that carbachol- and baclofen- induced inhibition of adenylyl cyclase was unaffected by a 48 hour exposure to 10 μM cyclopentyladenosine. Expression of G-protein α-subunits was not dramatically affected by agonist exposure. The fraction of sequestered A1 adenosine receptors was increased significantly at 4, 24 and 48 hours of cyclopentyladenosine exposure (35%, 57% and 81% increase over control, respectively). The time course of the agonist-induced A1 adenosine receptor sequestration was much slower than that reported for other G-protein coupled receptors. Incubation with the adenosine receptor antagonist, 8-para-sulfophenyltheophylline or adenosine deaminase (to remove endogenous adenosine) did not produce significant changes in sequestration at any time point assayed. Neither the steady state level of A1 adenosine receptor mRNA nor transcript stability were affected by exposure to agonist for 48 hours. We have determined that the half-life of A1AR in cerebellar granule cells is 20.9 hours which is considerably longer than that reported for several other G-protein coupled receptors. The slow time course of A1 adenosine receptor sequestration as well as the stability of the corresponding mRNA may be a reflection of the tonic inhibitory tone exerted by adenosine in brain.

3.2. Introduction

The ubiquitous neuromodulator, adenosine, elicits various physiological responses at a number of targets including brain, heart, kidney, adipocytes and vasculature (Olah and Stiles, 1995). The effects of adenosine are
mediated by A₁, A₂ₐ, A₂ₐ, and A₃ adenosine receptors (A₁AR, A₂ₐAR, A₂ₐAR, A₃AR) which are members of the family of G-protein coupled receptors. High expression levels of A₁AR are found in brain, spinal cord, fat and testis with lower levels in heart and kidney (Olah and Stiles, 1995). A₁AR are coupled to inhibition of adenylyl cyclase, activation of inward-rectifying potassium channels, inhibition of calcium currents and either activation or inhibition of phospholipase C (Linden, 1991).

Chronic activation of A₁AR and other G-protein coupled receptors leads to an attenuation of the cellular response to further agonist stimulation (Bohm, et al., 1997). This phenomenon, termed desensitization, provides a means for cells to regulate responsiveness to hormone and neurotransmitter stimuli and thereby maintain homeostasis at the cellular level. Mechanisms of receptor desensitization are manifold and include phosphorylation, sequestration, downregulation, uncoupling of receptors from G-proteins, downregulation of G-proteins and regulation of receptor and/or G-protein mRNA transcription or stability (Bohm, et al., 1997). The effects of chronic agonist exposure on A₁AR signal transduction have been examined in intact animals, cell lines endogenously expressing A₁AR and mammalian cell lines transfected with recombinant A₁AR (Olah and Stiles, 1995). We and others have shown that in neuronal (Fernandez, et al., 1995; Hettinger-Smith, et al., 1996), cardiac (Blair, et al., 1989; Shyrock, et al., 1989; Liang and Donovan, 1990; Lee, et al., 1993; Dennis, et al., 1995) and adipocyte (Parsons and Stiles, 1987; Longabaugh, et al., 1989; Green, et al., 1990, 1992) cells, as well as the smooth muscle cell line, DDT, MF-2 (Ramkumar, et al., 1991), chronic exposure to agonist produced a decrease in receptor density with no change in affinity of the receptor for an antagonist radioligand. A decrease in the coupling of A₁AR to G-proteins (Longabaugh, et al., 1989; Liang and Donovan, 1990; Lee, et al., 1993; Dennis, et al., 1995) and desensitization of agonist-induced inhibition of adenylyl cyclase (Hoffman, et al., 1986; Parsons and Stiles, 1987; Longabaugh, et al., 1989; Shyrock, et al., 1989; Ramkumar,
et al., 1991; Hettinger-Smith, et al., 1996) and lipolysis (Hoffman, et al., 1986; Longabaugh, et al., 1989; Shyrock, et al., 1989), as well as agonist-induced negative chrono-, ino- and dromotropy (Blair, et al., 1989; Shyrock, et al., 1989; Liang and Donovan, 1990; Lee, et al., 1993) in cardiac tissue have been documented in response to chronic activation of A\textsubscript{1}AR. Expression of A\textsubscript{1}AR mRNA was examined by in situ hybridization by Fernandez, et al. (1995) and no significant changes in transcript levels due to chronic infusion of rats with an A\textsubscript{1}AR agonist were found in any of the brain regions investigated. In CHO cells stably transfected with the human A\textsubscript{1}AR, chronic agonist exposure produced an increase in A\textsubscript{1}AR sequestration (Bhattacharya and Linden, 1996) as well as a decrease in the ability of the A\textsubscript{1}AR selective agonist cyclopentyladenosine (CPA) to inhibit adenylyl cyclase.

In addition to agonist-induced effects on A\textsubscript{1}AR, regulation of G-protein levels in response to chronic agonist exposure has been reported by several groups. In rat adipocytes (Parsons and Stiles, 1987; Lonagabaugh, et al., 1989; Shyrock, et al., 1989; Green, et al., 1990) and ventricular membranes (Lee, et al., 1993), as well as guinea pig atrial membranes (Dennis, et al., 1995), levels of inhibitory G-proteins were shown to decrease in response to chronic agonist exposure. Decreases of 90%, 50% and 90% of G\(_{\alpha}1\), G\(_{\alpha}2\) and G\(_{\alpha}3\), respectively, in agonist treated adipocytes were reported by Green, et al. (Green, et al., 1990); however, Longabaugh, et al. found decreases in G\(_{\alpha}1\) and G\(_{\alpha}2\), but not G\(_{\alpha}3\) in these same cells (Longabaugh, et al., 1989). In contrast, Ramkumar, et al. (1991) reported no change in G\(_{\alpha}1\) or G\(_{\alpha}2\) with chronic agonist exposure in DDT\(_1\), MF-2 cells.

Endogenous adenosine has been suggested to have a neuromodulatory role which manifests as a tonic inhibitory control exerted in the central nervous system (CNS). Within the CNS, adenosine receptors are heterogeneously expressed at high density (Goodman and Snyder, 1982). We have utilized primary cultures of cerebellar granule cells as a model to assess the mechanisms involved in regulation of A\textsubscript{1}AR in neural tissue. Our
earlier experiments demonstrated a cAMP independent decrease in A1AR density and coupling to G-proteins in response to chronic exposure to CPA resulting in a blunted agonist-induced inhibition of adenylyl cyclase (Hettinger-Smith, 1996). In the present study, we sought to understand further the mechanisms underlying the effects of chronic agonist exposure on A1AR in cerebellar granule cells. The blunted agonist-induced inhibition of adenylyl cyclase in cultures chronically exposed to CPA was found to be specific to A1AR. This homologous regulation involved no significant alterations in G-protein α-subunits. Chronic agonist exposure did however result in a significant increase in the fraction of sequestered A1AR with a delayed time course compared to other G-protein coupled receptors. Lastly, the reduction in the density of A1AR in response to chronic agonist exposure did not result from a decrease in A1AR mRNA.

3.3 Materials And Methods

3.3.1. Materials

[^H]1,3-dipropyl-8-cyclopentyl xanthine ([^H]DPCPX), [^H]adenine, [^14]C cyclic AMP ([^14]C cAMP), anti-Gα1,2, anti-Gα3, anti-Gqα, anti-Gsα were purchased from NEN-Dupont (Boston, MA). Antibodies to Gqα (C-19) and Gβ (T-20) and anti-rabbit IgG-horseradish peroxidase were from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Trypsin, DNase, trypsin inhibitor, glutamine, polylysine, gentamycin, fetal bovine serum, cytosine arabinoside (araC), glucose, forskolin, xylene cyanole, bromophenol blue and Triton X-100 were from Sigma Chemical Company (St. Louis, MO). N6-cyclopentyl-adenosine (CPA), 8-parasulfophenyltheophylline (8-pSPT), 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), carbachol and baclofen were from Research Biochemicals, Inc. (Natick, MA). Adenosine deaminase was from Boehringer Mannheim (Mannheim, Germany). Basal Eagle’s media was from Gibco (Gaithersburg, MD). Cytoscint scintillation cocktail was from ICN
Radiochemicals (Irvine, CA). Dowex A6 50W-X4, acrylamide and nitrocellulose membranes were from Biorad (Richmond, CA). The plasmid pBLUESCRIPT was from Stratagene (La Jolla, CA). Enhanced Chemiluminescent Western Blot detection solutions were purchased from Amersham (Arlington Heights, IL). TRIreagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). RNase T1, cyclophilin antisense probe template, yeast RNA, and RNA century markers were purchased from Ambion (Austin, TX). Restriction enzymes, MMLV reverse transcriptase, RQ1 DNase, RNasin, rNTPs and T7 RNA polymerase were obtained from Promega (Madison, WI). RNaid kit was purchased from Bio101 (Vista, CA). Other molecular biology-grade, common reagents were from Fisher (Pittsburgh, PA).

3.3.2. Preparation Of Cerebellar Granule Neurons

Rat cerebellar granule neurons were prepared from 8-day old Sprague Dawley rat pups as described previously (Hettinger-Smith, 1996; Novelli, et al., 1988). Dissection of cerebella was followed by dissociation with trypsin in the presence of DNase followed by addition of trypsin inhibitor to terminate digestion. Cells were physically dissociated and suspended in depolarizing media containing basal Eagle’s media, 25 mM KCl, 2 mM glutamine, 10% fetal bovine serum and 100 μg/ml gentamycin. Cells were then plated on polylysine coated (10μg/ml) 100 mm tissue culture dishes or 6-well, 35 mm dishes (for adenylyl cyclase assays and whole cell binding). AraC (10 μM) was added to the culture medium at 16-24 hours post-culture to limit replication of non-neuronal cells. Cells were maintained at 37°C with 5%CO₂/95% air and 95% humidity. CPA and 8-pSPT stocks (100X) were prepared in distilled water, sterile filtered and added to cell culture media on day 5 in culture.
3.3.3. Cerebellar Granule Cell Membrane Preparation And Binding Of \[^3\text{H}\]DPCPX

Cells were rinsed three times with phosphate buffered saline (PBS) to remove media and drugs prior to membrane preparation as described previously (Hettinger-Smith, 1996). Briefly, cells were collected and Dounce homogenized in 50 mM Tris-HCl, pH 7.5 at 4°C, followed by two washes of the pellets with 50 mM Tris-HCl, 1 mM EDTA, pH 7.5 at 4°C. This final pellet was resuspended in 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 at 22°C and incubated 2 hours with 2.5 U/ml adenosine deaminase to remove endogenous adenosine. Membranes were collected by centrifugation and resuspended for binding assays in 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 at 22°C with 0.5 U/ml adenosine deaminase. Granule cell membrane specific binding of \[^3\text{H}\]DPCPX (an A\(_1\)AR selective antagonist) was determined using a rapid filtration assay that has been described elsewhere (Blair, et al., 1989). Briefly, cerebellar granule cell membranes (50-100 µg protein) were incubated for 90 minutes at 22°C with \[^3\text{H}\]DPCPX in the absence or presence of 100 µM CPA (to define nonspecific binding). Equilibrium binding reactions were terminated by vacuum filtration using a Brandel cell harvester (M-48R) and polyethyleneimine-saturated Whatman GF/C filters. Liquid scintillation counting (Beckman Model LS 6000SC) was used to analyze filter-bound radioactivity. Membranes were incubated with 8-12 concentrations of \[^3\text{H}\]DPCPX (from 0.1 to 7 nM) for saturation binding experiments. A nonlinear regression program within Graph Pad Prizm software was used to analyze binding data. Membrane protein content was assayed by the method of Lowry, et al. (1951) after solubilization of the samples in 0.5 N NaOH. Bovine serum albumin (BSA) was used as the standard.
3.3.4. **Adenylyl Cyclase Assay**

Activity of adenylyl cyclase in intact cerebellar granule cells was assayed as per Salomon (1979) with minor modifications as previously described (Hettinger-Smith, 1996).

3.3.5. **Western Blotting**

Membranes from treated and untreated cells were collected as for radioligand binding excluding the adenosine deaminase incubation. Membrane pellets were resuspended in a small volume of 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 at 22°C and the protein concentration was determined as described above. Membrane samples (100 µg protein per lane) were resuspended in 2X sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 3.2% sodium dodecyl sulfate (SDS), 0.075% (w/v) bromophenol blue, 10% β-mercaptoethanol) and resolved on 12.5% SDS-polyacrylamide gels containing 4 M urea. Proteins were transferred to nitrocellulose filters (0.2 µM, Biorad) using a Biorad mini-Transblot apparatus. The nitrocellulose filter was blocked for 1 hour in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) containing 5% nonfat dry milk (NFDM). Incubations with primary antibodies (1:750 dilution of Gα1,2 [AS/7] and Gα3 [EC/2], 1:500 dilution of Gzα [RM/1], 1:1000 dilution of Gzα [GC/2], 1:2000 dilution of Gqα [C-19] and Gβ [T-20]) in TBS-T containing 5% NFDM were carried out at 4°C overnight with shaking. Blots were washed three times in TBS-T and once with TBS-T containing 5% NFDM. The secondary antibody (anti-rabbit IgG coupled to horseradish peroxidase) was diluted 1:1000 in TBS-T with 5% NFDM and incubated with the blots for 2 hours at room temperature with shaking. Visualization of specific G-protein α subunit bands by enhanced chemiluminescence (ECL) was performed per manufacturer’s instructions (Amersham) and quantitated by densitometry.
3.3.6. Whole Cell Binding Assay

Cells were plated in 6-well culture dishes (35 mm wells) and exposed to vehicle, CPA (10 μM), 8-pSPT (100 μM) or adenosine deaminase (ADA, 10 U/ml) for the indicated times. Cells were rinsed with ice cold Locke’s buffer (8.6 mM HEPES hemisodium salt, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1 mM MgCl₂, 2.3 mM CaCl₂). Cells were incubated for 4 hours on ice at 4°C in the presence of cold Locke’s buffer containing approximately 1 nM [³H]DPCPX, 2 U/ml adenosine deaminase and either vehicle (0.5% DMSO), 100 μM 8-pSPT (to define cell surface binding) or 1 μM unlabeled DPCPX (to define total cellular binding). Unbound [³H]DPCPX was removed by rinsing cells three times with ice cold PBS. Cells were then lysed with 750 μl 1% Triton X-100 and [³H]DPCPX in the resulting lysate was determined by liquid scintillation counting.

3.3.7. RNA Isolation And Preparation Of Riboprobes

Total RNA was isolated from cerebellar granule cell cultures using the single-step TRI reagent (Molecular Research Center) method as per manufacturer’s instructions.

Total RNA was prepared from whole rat brain and first strand cDNA was synthesized from RNA by reverse transcription using MMLV reverse transcriptase and random hexamer reverse transcription primers. Using the first strand cDNA as a template, the entire coding sequence of A₇AR was amplified by PCR and subcloned into the expression vector pTL1 (Leid, et al., 1992; gift from Dr. T. Lufkin). This clone was subjected to sequence analysis and found to be identical to that previously reported for rat brain A₇AR. A 219 base pair fragment of this rat A₇AR cDNA was amplified by polymerase chain reaction using a 5’ primer (5’-ATGGATCCATGTCATAAGCCTGATTGAGGCCTGACACCCAT-3’) containing a BamHI site (underlined), 15 base pairs on non-annealing random sequence and sequences corresponding to
the rat \(\text{A}_1\)AR cDNA (base pairs 405 to 425 of the coding sequence, bold) and a 3' primer (5'-ATGAATTCTAATACGACTCAGTATTACGGATCAGGTTACGGATAG AAGACC-3') containing an EcoRI site (underlined), non-annealing random sequence and rat \(\text{A}_1\)AR cDNA (base pairs 604 to 624 of the coding sequence, bold) complementary sequences. This fragment was subcloned into the expression vector pTL1 (Leid, et al., 1992; gift from Dr. T. Lufkin) which contains a T7 RNA polymerase binding site. The cDNA was linearized by BamHI digestion 5' to the insert and uniformly radiolabeled antisense riboprobes were synthesized with T7 RNA polymerase in a 50 µl reaction containing 1.5 µg linearized plasmid, 10 µl 5X transcription buffer, 4 mM MgCl\(_2\), 30 mM Tris, pH 8.0, 10 mM dithiothreitol, 0.34 mM each of rATP, rGTP, rUTP, 20 U RNasin, 10 U T7 RNA polymerase and 6 µl 800 Ci/mmole \(\alpha^{[32P]}\)-CTP. Antisense radiolabeled cyclophilin control probes were produced using the same reaction conditions with some exceptions: the digested template yielding a 103 base pair probe was purchased from Ambion, 0.2 mM rCTP was included in the reaction and only 2 µl 800 Ci/mmole \(\alpha^{[32P]}\)-CTP was used. Transcription reactions were incubated 90 min. at 37°C followed by digestion of plasmid DNA with 1.5 U RQ1 DNase for 15 min. at 37°C. The riboprobes were purified using a RNaid kit (Molecular Research Center, Inc.) as per manufacturer’s instructions and the incorporation of radioactivity was determined by liquid scintillation counting. \(\text{A}_1\)AR mRNA, prepared by in vitro transcription, was used as a control for all RNase protection assays.

### 3.3.8. RNase Protection Assay

The protocol for RNase protection assays was modified from the method of Gilman (1992). Total cerebellar granule cell RNA (50 µg) or control yeast RNA (50 µg) was precipitated with 150,000 cpm of \(\text{A}_1\)AR antisense probe and 50,000 cpm cyclophilin antisense probe using NH\(_4\)OAc and 100% ethanol. The precipitate was resuspended in 30 µl hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8, 0.4 M NaCl and 80% formamide) and
incubated at 85°C for 10 minutes to denature the RNA. Samples were transferred to a 50°C water bath for hybridization overnight (12 hours). Samples were cooled to room temperature, 300 μl RNase digestion mixture (300 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, pH 8 and 800 U RNase T1) was added and samples were digested for 1 hour at 30°C. The digestion reactions were terminated by addition of 330 μl guanidium solution (4M guanidium thiocyanate, 20 mM sodium acetate, 0.1 mM DTT, 0.5% sarkosyl, pH 5.5) and the samples were precipitated by addition of 25 μg yeast RNA and 650 μl isopropyl alcohol. Samples were centrifuged at 13,000 rpm in a microcentrifuge for 15 min. and the supernatants were removed and the pellets were air dried. Dry pellets were resuspended in 5 μl formamide loading solution (80% formamide, 10 mM EDTA, pH 8, 1 mg/ml xylene cyanole, 1 mg/ml bromophenol blue) and heated for 5 minutes at 95°C prior to electrophoresis through a 6% polyacrylamide/8M urea denaturing gel run in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Gels were dried and protected fragments were detected by autoradiography and quantitated by densitometry.

The half-life of A1AR mRNA was determined by incubation of cerebellar granule cell cultures with actinomycin D to block transcription (Reutter, et al., 1997). Actinomycin D (10 μg/ml) was added to cultures prior to addition of vehicle or 10 μM CPA. Total cellular RNA was extracted at 0, 12, 24 and 48 hours after CPA addition and A1AR mRNA levels were quantified by RNase protection assay as described above.
3.4. Results

3.4.1. Downregulation Of A₂AR Due To Chronic Agonist Exposure Corresponds To Homologous Desensitization Of Agonist-Induced Inhibition Of Adenylyl Cyclase

The downregulation of A₂AR produced by a 48 hour incubation with 10 μM CPA is depicted in Figure 1A. The density of receptors in CPA treated cell membranes was decreased by 63% relative to control (vehicle, 176.0 ± 10 fmol/mg protein and CPA, 65.7 ± 14.1 fmol/mg protein). In order to determine whether this decrease in receptor density was associated with a desensitization of A₂AR function, adenylyl cyclase activity was measured in vehicle and CPA treated cells. Activation of A₂AR is coupled to the inhibition of adenylyl cyclase via inhibitory G-proteins. Vehicle treated cerebellar granule cells demonstrated a concentration-dependent decrease in forskolin-stimulated adenylyl cyclase activity in response to agonist. The 48 hour CPA exposure blunted the effect of agonist on adenylyl cyclase (Figure 1B) with a 2.8-fold decrease in the ability of CPA to inhibit adenylyl cyclase (maximal inhibition 41.1 ± 2.7% and 14.6 ± 2.6% for vehicle and CPA treated cells, respectively). The EC₅₀ value for adenylyl cyclase inhibition in CPA treated cells (2.0 nM, 95% confidence interval (CI): 0.3 - 13.5 nM) was not significantly different from that of control cells (14 nM, 95% CI: 7.3 - 27 nM). Examples of both homologous and heterologous desensitization have been demonstrated for G-protein coupled receptors. Therefore, to address the nature of the observed regulation, we assessed the inhibition of adenylyl cyclase by the muscarinic acetylcholine receptor agonist, carbachol, and the GABA₉ receptor agonist, baclofen. As shown in Figures 1C and 1D, a 48 hour exposure to CPA did not affect the maximal inhibition of adenylyl cyclase by carbachol- (vehicle 80.9 ± 2.2% and CPA 82.6 ± 2.2%) or baclofen- (vehicle 87.7 ± 5.1% and CPA 90.2 ± 4.1%). The maximal inhibition of adenylyl cyclase afforded by carbachol and baclofen may be greater than that of CPA.
Fig. 10. Effect of CPA exposure on saturation binding of [3H]DPCPX and adenylyl cyclase inhibition of CPA, carbachol and baclofen. (A) [3H]DPCPX saturation binding in vehicle and 10 µM CPA treated cerebellar granule cell membranes. Squares and triangles represent binding in vehicle and CPA treated cells, respectively. The data shown are derived from the mean of triplicate data points from a representative experiment. The fit shown was obtained using nonlinear regression analysis which yielded a $K_D$ of $0.98 \pm 0.16$ nM and a $B_{max}$ of $176.0 \pm 10.0$ fmol/mg protein for vehicle treated cells and a $K_D$ of $0.91 \pm 0.56$ nM and a $B_{max}$ of $65.7 \pm 14.1$ fmol/mg protein for CPA treated cell membranes. (B) Decreased sensitivity of CPA-induced inhibition of AC activity due to chronic CPA exposure. Data are means of triplicate determinations from five experiments. The maximal inhibition of AC in vehicle treated cells was $41.1 \pm 2.7\%$ with an $EC_{50}$ of $14.0$ nM (95% CI: 7.3 - 27 nM) whereas the maximal inhibition of AC activity in 10 µM CPA treated cells was $14.6 \pm 2.6\%$ with an $EC_{50}$ of $2.0$ nM (95% CI: 0.3 - 13.5 nM). (C,D) Lack of effect of CPA exposure on carbachol- (C) and baclofen- (D) induced inhibition of AC activity. Data are means of triplicate determinations from three experiments. The maximal carbachol-induced inhibition of AC activity in vehicle treated cells was $80.9 \pm 2.2\%$ with an $EC_{50}$ of $1.1$ µM (95% CI: 0.80 - 1.5 µM) whereas in CPA treated cells inhibition of AC by carbachol had a maximum of $82.6 \pm 2.2\%$ with an $EC_{50}$ of $0.8$ µM (95% CI: 0.6 - 1.2 µM). Maximal inhibition of AC by baclofen was $87.7 \pm 5.1\%$ and $90.2 \pm 4.1\%$ with an $EC_{50}$ of $0.7$ µM (95% CI: 0.4 - 1.2 µM) and $1.0$ µM (95% CI: 0.6 - 1.5 µM) for vehicle and CPA treated cells, respectively.
due to a higher density of muscarinic acetylcholine and GABA<sub>B</sub> receptors compared to A<sub>i</sub>AR in cerebellar granule cell cultures. For example, muscarinic acetylcholine receptors were determined to be present at a density of 1.85 pmole/mg in cerebellar granule cell cultures as assessed by [3H]-N-methylscopolamine binding (Alonso, et al., 1990). The EC<sub>50</sub> values were also not significantly altered for carbachol- (vehicle treatment: 1.1 μM, CPA treatment: 0.8 μM) and baclofen- (vehicle treatment: 0.7 μM, CPA treatment: 1 μM) induced inhibition of adenylyl cyclase. This inability of chronic CPA exposure to alter inhibition of adenylyl cyclase by agonists of other G<sub>i</sub> coupled receptors indicates that the observed regulation of the A<sub>i</sub>AR-adenylyl cyclase signaling system is homologous in nature.

### 3.4.2. Regulation Of G-Protein Levels Does Not Play A Major Role In The Downregulation And Desensitization Of A<sub>i</sub>AR

A<sub>i</sub>AR have been shown to couple to downstream effectors via members of the G<sub>i</sub>/G<sub>o</sub> group of G-proteins (Olah and Stiles, 1995). Inasmuch as persistent adenosine receptor activation has been shown to decrease the levels of G-protein α-subunits in adipocytes and cardiac myocytes (Parsons and Stiles, 1987; Lonagabaugh, et al., 1989; Shyrock, et al., 1989; Green, et al., 1990; Lee, et al., 1993; Dennis, et al., 1995), we assessed the effect of CPA on G-proteins in cerebellar granule cells. Membranes from cells treated with CPA or 8-pSPT were collected and analyzed by Western blot using a series of G-protein α-subunit specific antibodies. There were no significant differences between immunoblots of control and CPA treated cell membranes using any of the G-protein α-subunit specific antibodies (Figure 2A) which suggests that alterations in G-proteins do not play a major role in desensitization of A<sub>i</sub>AR. CPA also produced no significant change in G<sub>q</sub>α and Gβ<sub>total</sub> (data not shown). These data are consistent with the homologous nature of the desensitization of adenylyl cyclase resulting from chronic CPA
Fig. 11. Summary of western blot analysis of G-protein α-subunits in cerebellar granule cells treated for 48 hours with vehicle, CPA or 8-pSPT. 10 µM CPA, 100 µM 8-pSPT or water was added to cells on day 5 in culture and membranes were prepared for Western blots as described in Methods. (A) Effect of CPA exposure on G-protein α-subunits. (B) Effect of 8-pSPT exposure on G-protein α-subunits. Western blots were probed with polyclonal antibodies specific for Gα1,2 (triangles and squares, respectively), Gα3 (inverted triangles), Gα (circles) and Gα (diamonds) followed by detection of specific bands by enhanced chemiluminescence. These graphs represent mean values and standard error from 3-6 experiments. Western blots were analyzed by densitometry of autoradiograms. Data are plotted as percent of control values. There were no statistically significant differences from the control value for any of the antibodies using an ANOVA.

exposure. In parallel experiments, chronic exposure to the adenosine receptor antagonist, 8-pSPT did not produce significant changes in the G-protein subunits assayed (Figure 2B and data not shown for Gqα and Gβ( total)).

3.4.3. Agonist Exposure Increases The Fraction Of Sequestered A1AR

Sequestration has been documented to play a critical role in both desensitization and resensitization for a number of G-protein coupled receptors. We have utilized a whole cell binding assay to evaluate sequestration of A1AR in cerebellar granule cells. Cells were incubated with
[³H]DPCPX and either 8-pSPT or unlabeled DPCPX as competitors to assess cell surface and total pools of receptors, respectively. The adenosine receptor antagonist 8-pSPT is hydrophilic and should not cross the cell membrane, thereby binding solely to membrane bound A₁AR (Daly, 1982). In contrast, DPCPX is lipophilic and permeable to the cell membrane and will therefore compete for binding sites that are both membrane bound and internalized. To prevent recycling of the sequestered receptors to the cell membrane, binding reactions were performed at 4°C. Initially, we determined the time required for the binding to reach equilibrium at this temperature (Figure 3A). Specific binding in intact neurons reached equilibrium by 4 hours and thus the remainder of the whole cell binding assays incorporated an incubation of this duration.

To characterize the equilibrium saturation binding of [³H]DPCPX in intact neurons, we incubated cells with increasing concentrations of [³H]DPCPX in the presence or absence of unlabeled DPCPX. The equilibrium binding of [³H]DPCPX was saturable and of high affinity; the affinity for [³H]DPCPX labeling of the total A₁AR population in intact neurons was 0.91 ± 0.11 nM.

Fig. 12. Characterization of whole cell binding assay in cerebellar granule cells. (A) Time course of specific (closed symbols) and nonspecific binding (open symbols) of [³H]DPCPX to intact cerebellar granule cells using 1 μM DPCPX to define nonspecific binding. This graph is a representative
experiment which was performed three times in triplicate. (B) Saturable binding of \[^{3}H\]DPCPX in intact cerebellar granule cells. The B_{\text{max}} was 3393 ± 142 specific CPM and K_{0} was 0.91 ± 0.11 nM when nonspecific binding was defined with unlabeled DPCPX. This graph is a representative experiment performed in triplicate three times.

To assess the influence of A_{1}AR activation on sequestration of membrane receptors, cerebellar granule cell cultures were incubated with 10 μM CPA, 100 μM 8-pSPT or 5 U/ml adenosine deaminase (ADA) for 0.5, 1, 2, 4, 24 and 48 hours and assayed for sequestration of A_{1}AR. Following incubation for 4, 24 or 48 hours, CPA exposure produced a significant increase in the fraction of A_{1}AR sequestered (Figure 4, diagonal bars). The control levels of sequestered receptors varied from 14 - 16% and after 4 hours of CPA exposure there was a significant increase in sequestered A_{1}AR to 21.8 ± 1.8% (p ≤ 0.05). At 24 and 48 hours, CPA also induced a significant increases of sequestration (fraction sequestered: 0.248 ± 0.026 and 0.260 ± 0.041 for CPA treated cells at 24 and 48 hours, respectively, and 0.158 ± 0.017 and 0.144 ± 0.029 for control treated cells at 24 and 48 hours, respectively; p ≤ 0.05). Interestingly, this agonist-induced increase in A_{1}AR sequestration proceeds over a much slower time course than that for other G-protein coupled (Eason and Liggett, 1992; von Zastrow and Kobilka, 1992; Palmer, et al., 1994; Pippig, et al., 1995).

Although not statistically significant, incubation of cerebellar granule cells with the adenosine receptor antagonist, 8-pSPT, appeared to decrease the fraction of A_{1}AR sequestered, notably at the 48 hour time point. This finding would be consistent with our previous results, demonstrating that antagonist exposure does increase the density of \[^{3}H\]DPCPX labeled A_{1}AR in cerebellar granule cell membranes (Hettinger-Smith, et al., 1996). We also tested the effect of adenosine deaminase incubation on sequestration (Figure 4, hatched bars). Adenosine deaminase was added to remove endogenous
adenosine from the culture media during the indicated exposure times. If extracellular adenosine was responsible for a basal rate of sequestration,

![Graph showing the effect of CPA, 8-pSPT, and ADA on sequestered AAR](image)

**Fig. 13.** Effect of CPA, 8-pSPT and adenosine deaminase (ADA) exposure on the fraction of sequestered AAR. Intact cells were incubated for 0.5, 1, 2, 4, 24 or 48 hours with either vehicle, 10 μM CPA, 100 μM 8-pSPT or 5 U/ml ADA. Whole cell binding was assayed by binding at 4°C for 4 hours in the presence of approximately 1 nM [³H]DPCPX and either vehicle, 100 μM 8-pSPT (to define cell surface binding) or 1 μM unlabeled DPCPX (to define total cellular binding). Cell surface binding was subtracted from total cellular binding to obtain a value representing internalized receptor binding. The ratio of internalized receptor binding to total receptor binding was used to represent the fraction of sequestered receptors. Control values are represented by solid bars, CPA by bars containing diagonal lines, 8-pSPT by unfilled bars and ADA bars containing hatched lines. This graph contains means and standard errors derived from four experiments. A * represents a value for fraction of sequestered receptors that is significantly different from the control for a given time point (p ≤ 0.05) as determined by ANOVA.
inclusion of adenosine deaminase should have reversed this effect. We did not, however, observe any significant change in the fraction of sequestered AβAR with adenosine deaminase treatment at any time point.

3.4.4. **Chronic Agonist Exposure Does Not Effect Steady State AβAR mRNA Levels Or Half-Life**

We ascertained whether the decrease in receptor protein due to chronic CPA exposure of cerebellar granule cells (Figure 1) was due to a decrease in AβAR transcript levels. Total RNA from cells treated with vehicle or 10 μM CPA for 0, 12, 24 or 48 hours was collected and RNase protection assays were used to determine the amount of specific AβAR mRNA that was present (Figure 5A). CPA exposure did not produce a significant change in the amount of AβAR mRNA present in cerebellar granule cells at any of the time points assayed (T=0 hr, 87 ± 8%; T=12 hr, 115 ± 8%; T=24 hr, 83 ± 9%; T=48 hr, 84 ± 9%; relative to time-matched control). To confirm the lack of effect of agonist exposure on AβAR mRNA, we examined the half-life of AβAR mRNA from vehicle and CPA treated cells. In these experiments, the transcriptional inhibitor, actinomycin D was added to cultures prior to addition of either vehicle or CPA. Total RNA was collected at 0, 12, 24 and 48 hours after addition of vehicle or CPA. A representative experiment is shown in Figure 5B. The rate of AβAR mRNA degradation in vehicle and CPA treated cells did not differ significantly as shown in Figure 5B, indicating that the CPA treatment did not effect the half-life of AβAR mRNA. The half-life for AβAR mRNA from vehicle treated cells was calculated to be 20.9 hours whereas the half-life for CPA treated cells was 26.2 hours. The half-life of AβAR mRNA determined in these experiments was considerably longer than the half-lives typically reported for other G-protein coupled receptors (Saitoh, et al., 1994; Hosoda, et al., 1995; Pippig, et al., 1995).
Fig. 14. Effects of CPA exposure on A₁AR mRNA in cerebellar granule cells. (A) Lack of significant effect of CPA exposure on steady state A₁AR mRNA levels. Cerebellar granule cells were exposed to vehicle or 10 μM CPA in the culture medium for the indicated times (in hours). A₁AR mRNA was measured by RNase protection assay using a 218 base pair antisense A₁AR probe. Resulting autoradiograms were quantified by image analysis and normalized to values obtained with a cyclophilin probe in each sample. Data points represent duplicates from a representative experiment performed three times. CPA treated cell A₁AR mRNA levels were not significantly different from time-matched control values at any time point as determined using a paired t-test. (B) Time course of A₁AR mRNA degradation in vehicle and CPA treated cells. A transcriptional inhibitor, actinomycin D (10 μg/ml) was added to cells just prior to vehicle or 10 μM CPA addition and remained in the culture media until collection of total RNA at the indicated time points. Levels of A₁AR mRNA were measured by RNase protection assay as described above. Vehicle treatment is represented by square symbols and a solid line whereas CPA treatment is represented by triangles and a dashed line. The half-life of A₁AR mRNA calculated from the slope of these lines was determined to be 20.9 and 26.2 hours for vehicle and CPA treated cells, respectively. The slopes of these lines were not significantly different (p = 0.55). This graph is a representative of three experiments with similar results.

3.5. Discussion

The results of these studies provide a characterization of the regulation of neuronal A₁AR utilizing cerebellar granule cell primary cultures as a model system. We have shown that a decrease in A₁AR density corresponds to a homologous regulation of the agonist-induced inhibition of adenylyl cyclase.
Previously, we have also demonstrated that a decreased efficiency of receptor-G-protein coupling also contributes to the blunted response of A1AR agonists on adenylyl cyclase (Hettinger-Smith, et al., 1996). Carbachol- and baclofen- induced inhibition of adenylyl cyclase via muscarinic acetylcholine and GABA_α receptors, respectively, was unaltered by chronic CPA exposure indicating that regulation at the G-protein level is unlikely to be the major mechanism for the observed homologous desensitization. In support of this observation, we determined that G-protein levels were not significantly affected by A1AR agonist exposure. This is in contrast to the results of Green, et al. (1990) and Longabaugh, et al. (1989) in which there was a dramatic reduction of G-protein α-subunits in adipocytes chronically exposed to the A1AR agonist, R-phenylisopropyladenosine (R-PIA). Green, et al. reported a 90% decrease in G_α1 and G_α3 with a more modest (50%) decrease in G_α2 (1990). The results of Longabaugh, et al. (1989) also demonstrated dramatic changes in specific G-protein α-subunits: G_α1 and G_α2 were decreased by 59% whereas G_α was increased by 49%. In contrast to the results of Green, et al. (1990), Longabaugh, et al. (1989) reported that G_α3 was unchanged due to chronic agonist exposure. In the smooth muscle cell line, DDT, MF-2, there was no change in G_α1, G_α2 or G_α as reported by Ramkumar, et al. (1991). The discrepancy in the ability of chronic agonist exposure to modulate G-protein levels in different tissues may represent a difference between G-protein levels in cerebellar granule neurons and peripheral tissues such as adipocytes and DDT, MF-2 smooth muscle derived cell line. G-protein levels are considerably higher in neuronal versus non-neuronal tissue (Brann, et al., 1987) rendering regulation of signal transduction at this level an unlikely mechanism, whereas the stoichiometry of G-proteins to receptors in peripheral tissues may be more amenable to regulation at this level.

The homologous nature of the regulation of functional responses coupled to activation of A1AR demonstrated herein does not appear to be universal. In adipocytes, adenosine agonist-induced desensitization of
adenylyl cyclase has been shown to be heterologous (Hoffman, et al., 1986; Parsons and Stiles, 1987; Green, et al., 1990) whereas, consistent with our results, intravenous administration of R-PIA in guinea pigs or chicks produced a homologous desensitization of the ability of adenosine receptor agonists to prolong AV nodal conduction time (Dennis, et al., 1995) or decrease tension of electrically paced right ventricular muscle strips (Shyrock, et al., 1989). These differences in the ability of chronic agonist exposure to affect G-protein levels and produce homologous versus heterologous desensitization suggest that regulation of the A₄AR signaling pathway is tissue specific.

To assess the effect of chronic agonist and antagonist exposure on sequestration of A₄AR, we used an intact cell binding assay utilizing membrane permeable (DPCPX) and impermeable (8-pSPT) adenosine receptor antagonists as a means to quantify total and membrane bound radiolabeled antagonist. The data presented in this study indicate that chronic agonist exposure of cerebellar granule cells increased sequestration of A₄AR. This increase in A₄AR sequestration did not occur with the same time course as has been shown for other members of the family of G-protein coupled receptors. Internalization of G-protein coupled receptors has been described extensively for β₂-adrenergic receptors (β₂AR) which display an agonist-induced increase in sequestered receptors that is maximal within 30 minutes and has a half-life of approximately 10 minutes (von Zastrow and Kobilka, 1992; Pippig, et al., 1995). These receptors are likely internalized by clathrin-mediated endocytosis (Goodman, et al., 1996) and it has recently been shown that one potential role of sequestration for β₂AR is resensitization of the receptors phosphorylated by GPCR kinases (GRK) (Pippig, et al., 1995; Krueger, et al., 1997). Ramkumar, et al. (1993) have recently demonstrated phosphorylation of the A₄AR by β-adrenergic receptor kinase which renders it possible that A₄AR sequestration, like that of β₂AR, may be required for receptor resensitization.
Interestingly, Palmer, et al. (1994) have recently documented an agonist-induced increase in sequestration for the adenosine A\textsubscript{2a} receptor, the extent of which is considerable within 30 minutes. Adenosine A\textsubscript{2a} receptors are coupled to the stimulation of adenyl cyclase and possess an additional 80-90 amino acids at the carboxyl-terminal tail of the protein (Olah and Stiles, 1995) relative to the A\textsubscript{1}AR sequence. One possible explanation for the distinct time courses of agonist-induced sequestration is the differential effect of receptors coupled to stimulation of adenyl cyclase (A\textsubscript{2a}AR and β\textsubscript{2}AR) versus receptors coupled to inhibition of adenyl cyclase (A\textsubscript{1}AR). However, other GPCR coupled to the inhibition of adenyl cyclase via G\textsubscript{i/0} subunits have been shown to display rapid agonist-induced internalization. Eason and Liggett (1992) demonstrated an agonist-induced increase in sequestration of the three subtypes of the α\textsubscript{2}AR (α\textsubscript{2c}10, α\textsubscript{2c}4 and α\textsubscript{2c}2) that was maximal within 30 minutes. Bhattacharya and Linden (1996) have also reported an increase in A\textsubscript{1}AR sequestration in response to a 24 hour exposure to CPA in stably transfected CHO cells although this report did not assay earlier time points. The slow time course of A\textsubscript{1}AR internalization in response to agonist likely reflects the role of adenosine as a tonic inhibitory neuromodulator in the CNS (Zhang, et al., 1993), rather than acting as a rapid neurotransmitter substance. Although extracellular adenosine concentrations may be elevated in the CNS in pathophysiologic states such as hypoxia and seizures, the purinergic inhibitory tone is thought to derive from the continuous presence of extracellular adenosine (Zhang, et al., 1993). This tonic effect of endogenous adenosine to suppress electrical activity in the CNS would be unlikely to be of significance if the A\textsubscript{1}AR was subject to rapid desensitization. Indeed, Dunwiddee and Fredholm (1989) have shown that there is no appreciable desensitization to the ability of A\textsubscript{1}AR agonists to inhibit synaptically evoked field excitatory postsynaptic potentials (epsp) despite the continuous presence of these compounds over the course of 10-20 minutes. The delayed appearance of agonist-induced sequestration of A\textsubscript{1}AR observed in the present
study is therefore consistent with the physiological role of adenosine in the CNS. Consonant with this interpretation, chronic $A_2$AR antagonist exposure or removal of adenosine from the medium by addition of adenosine deaminase to cultures both failed to affect sequestration indicating that basal adenosine levels did not exert a sufficient stimulus for promoting sequestration. The mechanism and function of $A_2$AR sequestration remain to be fully understood.

Several GPCR are regulated at the level of transcription or message stability (Collins, et al., 1989; Fukamauchi, et al., 1993; Saitoh, et al., 1994; Hosoda, et al., 1995). Chronic agonist exposure of the prototypic $\beta_2$AR has been demonstrated to result in a downregulation of receptor mRNA by 50% after two hours in C6 glioma cells (Hosoda, et al., 1995) and 24 hours in DDT, MF-2 cells (Collins, et al., 1989). The decrease in $\beta_2$AR mRNA was characterized as a decrease in transcription with no change in the half-life (2 hours) of the message (Hosoda, et al., 1995). The $G_i$ coupled $m_2$ muscarinic acetylcholine receptor in cultured cerebellar granule cells (Fukamauchi, et al., 1993) and $\alpha_{2A}$ adrenergic receptor ($\alpha_{2A}$AR) in rat astroglial cultures have also been reported to demonstrate agonist-induced decreases in receptor mRNA with chronic exposure (Reutter, et al., 1997). Reutter, et al. (1997) characterized this decrease in $\alpha_{2A}$AR message as a cAMP-mediated decrease in transcription with no significant effect on message stability. Conflicting results on the regulation of $A_{2A}$ adenosine receptors in response to chronic agonist stimulation have been reported by Saitoh, et al. (1994) and Fernandez, et al. (1995). In PC12 cells, Saitoh, et al. (1994) demonstrated a transient increase followed by a dramatic decrease of $A_{2A}$ adenosine receptor mRNA levels induced by the NECA. The decrease, measured by northern analysis, was mimicked by forskolin and was not due to significant reductions in message stability (Saitoh, et al., 1994). Alternatively, Fernandez, et al. (1995) reported that there were no significant changes in $A_{2A}$ adenosine receptor mRNA measured by in situ hybridization in brains from rats exposed to R-PIA for 7 days.
The results presented herein are similar to those of Fernandez, et al. (1995) for the $A_{2a}$AR in that chronic agonist exposure produced no significant change in $A_{1}$AR mRNA levels. We also measured message stability in treated and untreated cells and did not observe an effect of agonist exposure on the rate of degradation of $A_{1}$AR mRNA. Interestingly, the half-life we determined for the $A_{1}$AR mRNA in control cells was nearly 21 hours which is somewhat longer than that of many other GPCR; the half-life of the $\beta_{2}$AR, $\alpha_{2a}$AR and $A_{2a}$AR were determined to be 47 min (Ramkumar, et al., 1993), 3 hours (Reutter, et al., 1997) and 1.2 hours (Collins, et al., 1989), respectively. The slow degradation of $A_{1}$AR mRNA is on the order of that typically observed for stable transcripts and may facilitate continuous expression of $A_{1}$AR in the face of a continuous presence of agonist.

These studies demonstrate that the regulation of $A_{1}$AR involves a decrease in receptor density which underlies a homologous desensitization of agonist-induced inhibition of adenylyl cyclase. Regulation of G-protein levels does not accompany this desensitization, however, agonist exposure does increase the fraction of $A_{1}$AR sequestered. The downregulation of receptor number due to chronic agonist exposure is not due to alterations in the stability of $A_{1}$AR mRNA. In contrast to other GPCR, the time course of the agonist-induced $A_{1}$AR sequestration is prolonged, and the relatively long half-life of $A_{1}$AR mRNA indicates a stable message. These characteristics may reflect the unique modulatory role of endogenous adenosine as a tonic inhibitor of excitability in the CNS.

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3.7. References


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Chapter 4

Conclusions

Adenosine is a ubiquitous neuromodulator that exerts its actions at a number of targets. In the central nervous system, endogenous adenosine plays important roles in protection against stroke, epilepsy and neurodegenerative disorders via tonic inhibition mediated by $A_1$ adenosine receptors ($A_1$AR). We have utilized cerebellar granule cells as a model to assess the regulation of neural $A_1$AR in response to chronic agonist and antagonist exposure. We demonstrate that $A_1$AR regulation in response to chronic agonist and antagonist exposure reflects the unique modulatory role of endogenous adenosine.

Chronic exposure of cerebellar granule cells to adenosine receptor agonists results in a downregulation of receptors and an uncoupling of $A_1$AR from inhibitory G-proteins which underlies a reduced ability of adenosine receptor agonists to inhibit adenylyl cyclase. The desensitization of adenylyl cyclase in response to chronic receptor activation was homologous in nature and accordingly there was no significant change in inhibitory G-protein levels. The high expression of G-proteins in brain relative to other tissues may explain discrepancies between our data and demonstrated reductions in G-protein $\alpha$-subunits in peripheral tissues exposed to $A_1$AR agonists. In brain, an excess of G-proteins relative to $A_1$AR may make regulation at the level G-proteins inefficient.

We have demonstrated an increase in the fraction of sequestered $A_1$AR in response to chronic agonist exposure of neuronal cultures. The time course for this agonist-induced sequestration was slower than that reported for several other G-protein coupled receptors (GPCR). The slow time course of $A_1$AR internalization is consonant with the role of adenosine as a tonic
inhibitory neuromodulator in the CNS, rather than acting as a rapid neurotransmitter.

Unlike some members of the GPCR family, changes in A₁AR mRNA expression do not underlie the downregulation of A₁AR occurring in response to chronic agonist exposure. We have determined that A₁AR mRNA has a half life associated with stable transcripts and that this half-life is not altered by agonist exposure. The relative stability of A₁AR mRNA may facilitate continuous expression of A₁AR in the face of a continuous presence of agonist.

Chronic antagonist exposure of A₁AR results in an upregulation of receptors and increased coupling of A₁AR to inhibitory G-proteins which, in turn, produces a supersensitivity to agonist-induced inhibition of adenylyl cyclase. There was no significant concomitant increase in inhibitory G-proteins or agonist-induced sequestration in response to chronic antagonist exposure although there was a tendency towards a decreased fraction of sequestered receptors at longer antagonist exposure times. Antagonist-induced upregulation thus may act by relieving tonic downregulation of A₁AR in response to endogenous adenosine.

Adenosine has been suggested to be an endogenous neuro- and cardioprotectant consonant with the role of endogenous adenosine to exert tonic inhibition. The stability of A₁AR in response to endogenous adenosine levels is therefore of critical importance for maintenance of normal physiologic function. The clinical development of adenosine analogs and adenosine transport inhibitors for ischemia, epilepsy and tachycardia exploits the inhibitory nature of adenosine but must also take into account reduced responsiveness that may result from chronic activation of adenosine receptors.
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APPENDIX 1. Glucocorticoid Receptor Activation Leads To Upregulation Of A₁ Adenosine Receptors Whereas Activation Of Retinoic Acid Receptors (RAR) Had No Effect.

Upon activation by agonists, both glucocorticoid and retinoic acid receptors act to transcriptionally modulate a number of genes (as reviewed by Davies and Lefkowitz, 1984). Cerebellar granule cell cultures were exposed to the synthetic glucocorticoid, dexamethasone (Sigma, St. Louis, MO) and all-trans retinoic acid in order to assess the effects of activation of glucocorticoid and retinoic acid receptors, respectively, on neuronal A₁ adenosine receptor (A₁AR) expression.

Membranes derived from cultures treated with dexamethasone were assayed for binding of the radiolabeled A₁AR selective adenosine receptor antagonist [³H]DPCPX as described in materials and methods in chapters 2 and 3. Unlabeled N⁶-cyclopentyladenosine (CPA) was used to define nonspecific binding. Figure 1A, Appendix 1 demonstrates an increase in [³H]DPCPX binding with increasing duration of exposure to 10 nM dexamethasone. Binding in membranes from dexamethasone treated cells was significantly higher than control values at 6, 24, 48 and 72 hours (16%, 35%, 39% and 50%, respectively, p < 0.05). This upregulation of A₁AR was much more rapid than the antagonist-induced upregulation of A₁AR described in chapter 2. The dose-dependence of the upregulation of A₁AR by dexamethasone was investigated by incubation of cerebellar granule cells for 72 hours with increasing concentrations of dexamethasone (Figure 1B, Appendix 1). The EC₅₀ of this dose-dependent increase was 1.2 nM (95% confidence interval, 0.26 to 5.1 nM). Saturation binding experiments in membranes collected 72 hours post-dexamethasone addition were utilized to determine the effect of glucocorticoid activation on A₁AR density and affinity (Figure 1C, Appendix 1). Maximal binding (Bₘₐₓ) of [³H]DPCPX was increased by 29% with dexamethasone treatment (Bₘₐₓ = 152.4 ± 8.6 and 196.7 ± 7.0 fmol/mg protein for vehicle and dexamethasone treated cell membranes,
respectively) whereas the affinity was not significantly affected ($K_d$ 0.33 ± 0.06 and 0.37 ± 0.04 nM for vehicle and dexamethasone treatment, respectively). The magnitude of the response to dexamethasone likely indicates that glucocorticoid receptor activation does not act directly to activate transcription of A$_1$AR but may act indirectly. In support of this hypothesis, the analysis of the human A$_1$AR promoter by Ren and Stiles (1995) indicated the absence of specific response elements required for DNA recognition and transcriptional activation by glucocorticoid receptors. In the smooth muscle cell line, DDT$_1$ MF-2, Gerwins and Fredholm (1991) demonstrated a 54% increase in $[^3H]$DPCPX binding with no change in affinity of A$_1$AR. The reported EC$_{50}$ of this response was 1 nM which is in agreement with the data presented in Figure 1B, Appendix 1. The maximal upregulation of A$_1$AR in the DDT$_1$ MF-2 cells was achieved after 24 hours. The upregulation reported by Gerwins and Fredholm (1991) was prevented by addition of the protein synthesis inhibitor, cycloheximide which indicates a requirement for protein synthesis in glucocorticoid-mediated upregulation of A$_1$AR. The $\beta_2$-adrenergic receptor has also been demonstrated to be upregulated in response to dexamethasone treatment of DDT$_1$ MF-2 cells although the extent of this effect is much greater with a 2.2 ± 0.4 - fold increase in receptor number. This increase is paralleled by an increase in the rate of transcription representing a direct transcriptional effect of glucocorticoid receptor activation.

Figure 1D, Appendix 1 demonstrates the lack of effect on A$_1$AR binding parameters in membranes derived from neurons exposed to vehicle or 10 $\mu$M all-trans retinoic acid. These data indicate that retinoic acid receptor activated by all-trans retinoic acid does not regulate neuronal A$_1$AR.
Fig. 15. Effect of glucocorticoid and retinoic acid receptor activation on Aₑ adenosine receptor (A₁AR) binding in cerebellar granule cells (CGC). A) CGC were incubated with 10 nM dexamethasone (DEX) for the indicated exposure times, membranes were collected and binding of [³H]DPCPX was assayed. DEX treated membranes demonstrated 123 ± 8%, 116 ± 3%, 131 ± 11%, 135 ± 1%, 139 ± 2% and 150 ± 4% of [³H]DPCPX binding in membranes derived from vehicle treated cells at t=1,6,12,24,48 and 72 hours. A * represents a significant difference between vehicle and DEX treatments (p<0.05). At t=1 and 12 hours the increase in binding produced by DEX was marginally significant. B) CGC were incubated for 72 hours with increasing concentrations of DEX. The EC₅₀ of the dose-dependent increase in A₁AR was 1.16 nM (95% CI: 0.26-5.14 nM). The maximal increase was 33 ± 4%. C) Membranes derived from CGC treated with vehicle and DEX for 72 hours were incubated with [³H]DPCPX (0.09-3.39 nM) and bound [³H]DPCPX values were fit using nonlinear regression analysis in Graph Pad Prizm software. Squares and triangles represent values for vehicle and DEX treated CGC membranes, respectively. D) CGC were incubated with 10 μM all-trans retinoic acid (T-RA) for 72 hours and membranes derived from these cells were analyzed in saturation binding experiments. The B₅₀ for vehicle and T-RA treated cell membranes was 125.5 ± 6.4 and 124.7 ± 7.5 fmol/mg protein and the Kᵯ was 0.26 ± 0.05 and 0.24 ± 0.05 nM, respectively. Nonspecific binding was defined in the presence of 100 μM CPA.

2.1. Rationale

Sequestration is a critical component of desensitization and resensitization processes for a number of G-protein coupled receptors (GPCR). In efforts to determine which regions within GPCR are required for sequestration, several groups have investigated the importance of the carboxyl tail of the receptor and of a putative sequestration signal consisting of the amino acid sequence NPXXY, where X is any amino acid, which occurs in the seventh transmembrane spanning domain of the receptor.

Sequestration of many GPCR occurs in response to chronic agonist exposure. Internalization via clathrin coated pit mediated endocytosis or clathrin independent pathways (e.g. localization to caveolae) has been demonstrated (Tolbert and Lameh, 1996; Chun, et al., 1994; Roettger et al., 1995). The most thoroughly characterized GPCR sequestration pathway is that of the β2-adrenergic receptor (β2AR). Sequestration of β2AR in response to agonist exposure occurs in a time frame of minutes (Waldo, et al., 1983). Chronic agonist exposure of this short duration also results in phosphorylation and functional desensitization of the β2AR by GPCR specific kinases (GRK, i.e. βARK) (Benovic, et al., 1989). Ferguson, et al. (1995) demonstrated that phosphorylation of β2AR is not required for sequestration. In contrast, Moro, et al. (1993), Lameh, et al. (1992) and Tsuga, et al. (1994) have demonstrated that phosphorylation of muscarinic acetylcholine receptor m1 and m2 subtypes is required for agonist-induced sequestration. Yu, et al. (1993) and Pippig, et al. (1995) have recently determined that sequestration of the β2AR is involved in receptor dephosphorylation and thus resensitization.

Several investigators have attempted to elucidate whether there are specific molecular determinants within the β2AR and other GPCR that act to
target the receptor for internalization. An analysis of hybrid β2AR and β3AR (β3AR is resistant to short term agonist-induced desensitization and sequestration) has recently implicated multiple molecular determinants within the β2AR cytoplasmic domains as well as overall conformation to be important for sequestration (Jockers, et al., 1996).

Specific sequences for sequestration within the carboxyl terminal tail of GPCR have been documented and appear to be receptor-specific. Both μ and δ opioid receptor (μOR and δOR) carboxyl terminal truncation mutants have been analyzed with respect to a potential role in desensitization and sequestration (Segredo, et al., 1997; Trapaidze, et al., 1996). A truncation at amino acid 363 in the μOR displayed (D-Ala², N-Me-Phe⁴, Gly-ol⁵)-enkephalin (DAMGO)-induced internalization into clathrin and transferrin containing vesicles similar to the wildtype receptor. A second μOR mutant, truncated at amino acid 354 displayed constitutive internalization (Segredo, et al., 1997). Therefore, amino acids 354 to 363 of the μOR were proposed to play a regulatory role in agonist-induced sequestration. In contrast, truncation of the carboxyl terminal 15 or 37 amino acids of the δOR resulted in a significant reduction in the rate of receptor internalization (Trapaidze, et al., 1996). Point mutations at potential phosphorylation sites between serines 344 and 363 in the carboxyl tail were demonstrated to have reduced internalization in response to agonist (Trapaidze, et al., 1996) indicating an important role for δOR phosphorylation in sequestration.

Results from other mutagenesis studies suggest an inhibitory role in agonist-induced internalization for the carboxyl tail of GPCR. Truncation of the angiotensin II (AT₁A) receptor by 45 amino acids, including 13 serine and threonine residues, inhibits agonist-induced internalization (Thomas, et al., 1995). Benya, et al. (1993) determined that serines and threonines in the carboxyl tail of the gastrin-releasing peptide receptor were required for sequestration. Sequestration of the thyrotropin-releasing hormone receptor is dependent on two domains within the carboxyl terminus (Nussenzveig, et al., 1996).
1993) and truncation of the entire carboxyl tail of the yeast α mating factor receptor abolishes agonist-induced endocytosis (Reneke, et al., 1988). In contrast to these studies, Goldman, et al. (1996) demonstrated that the carboxyl terminal tail of the m<sub>2</sub> muscarinic acetylcholine receptor was neither necessary nor sufficient for receptor sequestration. Whether the removal of potential phosphorylation sites (serines and threonines) or deletion of some other sequestration signal is responsible for carboxyl terminal truncation reductions in internalization remains to be elucidated.

The consensus sequence NPXXY or NPXY in or near the seventh transmembrane spanning domain has been implicated as a critical signal for agonist-induced internalization for some, but not all, GPCR. Initially, a NPXY consensus sequence was recognized to be required for rapid internalization of the cell surface low density lipoprotein (LDL) receptor (Chen, et al., 1990). This carboxyl terminal tail sequence was proposed to be important for internalization of other cell surface proteins as well. Barak, et al. (1994, 1995) determined that a tyrosine residue at amino acid 326 proximal to the seventh transmembrane spanning domain of the β<sub>2</sub>AR was critical for agonist mediated sequestration. This group (Barak, et al., 1994) predicted that a consensus NPXXY (X is an aliphatic amino acid) present in human β<sub>2</sub>AR, D2 dopamine, m4 muscarinic, follicle stimulating hormone (FSH), rhodopsin and thrombin receptors, as well as bovine endothelin receptors may be critical for agonist-mediated receptor endocytosis. In a later report, Barak, et al. (1995) suggested that the NPLIY of β<sub>2</sub>AR may be critical for maintenance of the proper receptor conformation but not specifically as a sequestration recognition motif. In fact, Slice, et al. (1994) demonstrated that the NPX<sub>n</sub>Y motif of the gastrin-releasing peptide receptor is not required for sequestration. Furthermore, Hunyady, et al. (1995) determined that NPLFY was not required for angiotensin II receptor (AT<sub>1</sub>,<sub>a</sub>) sequestration but did contribute to both agonist binding and signal transduction. The putative role of
NPXXY motifs in GPCR agonist-mediated sequestration has therefore yet to be conclusively demonstrated.

The identity of domains involved in A\textsubscript{s}AR endocytosis have not been ascertained. We have therefore constructed several A\textsubscript{s}AR truncations as well as a tyrosine to alanine mutation at amino acid 288 to determine whether these sequences are important for A\textsubscript{s}AR sequestration. One of the truncation mutants (FLARA\textsubscript{1}AR\textsubscript{Δ}291) removes 35 of the 36 amino acids from the carboxyl terminus of the A\textsubscript{s}AR. FLAGA\textsubscript{1}AR\textsubscript{Δ}297 truncates the receptor by 29 amino acids which includes the only putative phosphorylation site of the A\textsubscript{s}AR COOH tail, threonine 298. FLAGA\textsubscript{1}AR\textsubscript{Δ}310 truncates the receptor by 16 amino acids containing a high number of prolines and acidic amino acids which could be important for protein-protein interactions. Site directed mutagenesis of tyrosine 288 to alanine (FLAGAIARY\textsubscript{288}A) targets the putative sequestration signal NPXXY. To easily assess expression of these mutants, we have introduced the FLAG epitope to the amino terminal end of all receptor constructs. All mutants were stably transfected into Chinese hamster ovary (CHO) cells in order to investigate their responses to chronic agonist exposure.

2.2. Construction of epitope-tagged wild type and mutant A\textsubscript{s}AR

2.2.1. Wild Type A\textsubscript{s}AR

Total RNA was isolated from adult rat brain by the method of Chirgwin, et al. (1979). First strand cDNA was synthesized from RNA by reverse transcription using MMLV reverse transcriptase. Using the rat first strand cDNA as a template, PCR amplification was performed with \textit{taq} polymerase and primers specific to the A\textsubscript{s}AR. The PCR product was subcloned into the expression vector pTL1 (Dr. T. Lufkin, Leid, et al., 1992) and designated pA\textsubscript{s}R-FL. This construct was used as a template for subsequent PCR amplification.
2.2.2. **Mutant A\textsubscript{2}ARs**

Primers designed to introduce a FLAG epitope 5' to the A\textsubscript{2}AR sequence and to truncate the A\textsubscript{2}AR at the indicated amino acids, are shown below.

**FLAG\text{A1AR}:** 5' - CCG GAA TTC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAA ATG CCG CCC TAC ATC TCG GCA - 3'

**A\textsubscript{1}AR\@291:** 5' - GAA CTT GTG GAT AAG CTT GAT ATC CTA CCG GAA GGC ATA GAC GAT GGG - 3'

**A\textsubscript{1}AR\@297:** 5' - TTC AGA AAG GTG AAG CTT CCC GGG CTA ACC CGG AAC TTG TGG ATC CGG - 3'

**A\textsubscript{1}AR\@310:** 5' - GGG AGG CTT GGG AAG CTT CTG CAG CTA CTG GCA TCG GAA GTG GTC ATT - 3'

**A\textsubscript{1}AR\@FL:** 5' - GGC CGC CTC GAC AAG CTT CTC GAG CTA GTC CTC AGC TTT CTC CTC TGG - 3'

The upstream 5' primer FLAG\text{A1AR} consists of an EcoRI site followed by the base pair sequence coding for methionine and amino acids of the FLAG epitope which is recognized by a commercially available anti-FLAG antibody (Sigma, St. Louis, MO) as well as the first 21 base pairs of the A\textsubscript{2}AR coding sequence. This upstream primer was used as the 5' primer for all PCR reactions, adding an epitope tag to wild type and mutant receptors.

The 3' primer for mutant FLAG\text{A1AR}\@291, A1AR\@291 contained A\textsubscript{2}AR base pair sequence corresponding to amino acids 285 to 291 followed by an in frame stop codon and EcoRV and HindIII restriction enzyme sites. PCR amplification with FLAG\text{A1AR} and A1AR\@291 produced a truncation mutant of A\textsubscript{2}AR with a 35 amino acid deletion representing most of the carboxyl tail. The 3' primer for mutant FLAG\text{A1AR}\@297, A1AR\@297 consisted of base pair sequence corresponding to amino acids 291 to 297 of A\textsubscript{2}AR followed by an in frame stop codon and restriction enzyme sites SmaI and HindIII. PCR amplification with FLAG\text{A1AR} and A1AR\@297 produced a truncation mutant missing the carboxyl 29 amino acids of A\textsubscript{2}AR which includes a threonine at
amino acid 298. The 3’ primer for mutant FLAGA1ARΔ310, A1AR@310 contained A1AR sequence corresponding to amino acids 304 to 310, an in frame stop codon and restriction enzyme sites, PstI and HindIII. PCR using FLAGA1AR and A1AR@310 amplified a fragment coding for an A1AR mutant truncated at amino acid 310 which removed 16 amino acids containing four prolines and seven acidic amino acids. The 3’ primer for FLAGA1ARFL (full length), A1AR@FL contained the carboxyl terminal 21 base pairs of A1AR followed by an in frame stop codon and Xhol and HindIII restriction enzyme sites and PCR amplification with this primer and FLAGA1AR produced a full length, epitope tagged A1AR construct.

All PCR products were digested with EcoRI and HindIII and ligated into an EcoRI/HindIII digest of the expression vector pTL1 which had been treated with calf intestinal alkaline phosphatase. All constructs were verified by sequencing.

2.2.3. Site-Directed Mutagenesis Of A1AR (FLAGA1AR-Y288A)

The full length FLAG tagged A1AR clone was used as the template for site directed mutagenesis using the Transformer™ site-directed mutagenesis kit (Clontech, Palo Alto, CA). A mutagenic primer (FLAGA1ARY288A) 5’-ATG AAC CCC ATC GTC GCT GCC TTC CGA ATC CAC AAG TTC CGG-3’ was designed that converted a TAT coding for tyrosine 288 to GCT coding for alanine and also converted the nearby BamHI site (GGATCC) to GAATCC. Removal of this BamHI site allowed for rapid screening of mutants. Site-directed mutagenesis was verified by sequencing of the mutant plasmid.

2.3. Transfection Of Wild Type And Mutant FLAGA1AR Constructs

20 μg of FLAGA1ARΔ291, FLAGA1ARΔ297, FLAGA1ARΔ310 FLAGA1AR-FL or FLAGA1AR-Y288A was transfected with 500 ng pRC-RSV (Invitrogen, contains a G418 resistance marker) into CHO cells using a calcium phosphate precipitation method (Chen and Okayama, 1987). CHO
cells (approximately 40% confluent) media (F12 media with 10% fetal bovine serum) was replaced with serum free media one hour prior to transfection. DNA and CaCl$_2$ were mixed and slowly added to 2X HEPES buffered saline (HBS, 274 mM NaCl, 10 mM KCl, 1.4 mM Na$_2$HPO$_4$, 11 mM dextrose, 42 mM HEPES acid, pH 7.05). These solutions were allowed to sit for 45 minutes at 22° C. Media was aspirated from CHO cells and the DNA/calcium phosphate precipitate was added. The precipitate was allowed to adsorb to cells for 20 minutes at 22°C. Media containing serum was added to cells and cells were incubated at 37°C for 4 hours. After 4 hours, media was aspirated from the cells and 15% glycerol in HBS was added to the cells, followed by a 4 minute incubation at 37°C. After thorough rinsing of cells with serum-free media, fresh media including 10% fetal bovine serum was added to the cells. After an approximately 36 hour incubation at 37°C, cells were split and grown in media containing G418 (700 μg/ml). Nine days later, individual clones were selected and grown in sufficient quantities to screen for expression of A$_1$AR constructs.

2.4. Screening

Individual clones were screened by binding of the A$_1$AR selective antagonist [³H]DPCPX and 10 μM CPA was used to define nonspecific binding. Twenty clones of wild type and each mutant receptor were screened and those with displaceable binding were further characterized by saturation binding analysis as described in chapters 2 and 3.

Figure 1, Appendix 2 shows representative saturation binding of wild type and mutant FLAG tagged receptors and Table 1, Appendix 2 summarizes maximal binding (B$_{max}$) and affinity (K$_D$) values obtained for each clonal cell line assayed by saturation binding.

In the preliminary screening experiment, none of the FLAGA1ARΔ291 clones demonstrated displaceable binding of [³H]DPCPX and were therefore not analyzed by saturation binding. The removal of the entire carboxyl
terminal tail in these mutant receptors may affect either their expression, targeting to the cell membrane or overall conformation required for antagonist binding. Untransfected CHO cells did not have significant displaceable $[^3H]$DPCPX binding.

**Fig. 16.** $[^3H]$DPCPX saturation binding in membranes derived from representative FLAGA1ARΔ297, FLAGA1ARΔ310 FLAGA1AR-FL or FLAGA1AR-Y288A expressing CHO cell lines (circles, diamonds, squares and inverted triangles, respectively). The fit shown was obtained using nonlinear regression analysis.
Table 1. Summary of saturation binding experiment in CHO cells transfected with epitope tagged wild type and mutant A1 adenosine receptors

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>$B_{\text{max}}$ (fmol/mg protein)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAGA1ARΔ297-19</td>
<td>297 ± 21</td>
<td>0.51 ± 0.12</td>
</tr>
<tr>
<td>FLAGA1ARΔ297-27</td>
<td>824 ± 82</td>
<td>0.56 ± 0.18</td>
</tr>
<tr>
<td>FLAGA1ARΔ297-31</td>
<td>425 ± 159</td>
<td>0.45 ± 0.46</td>
</tr>
<tr>
<td>FLAGA1ARΔ310-14</td>
<td>13620 ± 748</td>
<td>0.75 ± 0.11</td>
</tr>
<tr>
<td>FLAGA1ARΔ310-22</td>
<td>15200 ± 1749</td>
<td>1.20 ± 0.34</td>
</tr>
<tr>
<td>FLAGA1ARΔ310-25</td>
<td>4481 ± 132</td>
<td>0.72 ± 0.06</td>
</tr>
<tr>
<td>FLAGA1ARΔ310-47</td>
<td>2596 ± 189</td>
<td>0.92 ± 0.19</td>
</tr>
<tr>
<td>FLAGA1ARΔ310-48</td>
<td>5198 ± 244</td>
<td>1.01 ± 0.13</td>
</tr>
<tr>
<td>FLAGA1ARΔ310-49</td>
<td>65 ± 19</td>
<td>0.35 ± 0.28</td>
</tr>
<tr>
<td>FLAGA1AR-FL-8</td>
<td>913 ± 82</td>
<td>0.81 ± 0.21</td>
</tr>
<tr>
<td>FLAGA1AR-FL-52</td>
<td>3362 ± 247</td>
<td>0.64 ± 0.14</td>
</tr>
<tr>
<td>FLAGA1AR-FL-56</td>
<td>3013 ± 175</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>FLAGA1AR-FL-69</td>
<td>4314 ± 131</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>FLAGA1AR-FL-74</td>
<td>7501 ± 235</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td>FLAGA1AR-FL-76</td>
<td>7236 ± 565</td>
<td>0.77 ± 0.18</td>
</tr>
<tr>
<td>FLAGA1ARY288A-4</td>
<td>2928 ± 139</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>FLAGA1ARY288A-34</td>
<td>1129 ± 114</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>FLAGA1ARY288A-51</td>
<td>1141 ± 38</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>FLAGA1ARY288A-91</td>
<td>902 ± 57</td>
<td>0.44 ± 0.10</td>
</tr>
</tbody>
</table>

2.5. Future Directions

2.5.1. FLAGA1ARΔ291

The expression of FLAGA1ARΔ291 will be assessed by Western blot analysis of membrane and soluble fractions of each potential FLAGA1ARΔ291 expressing cell line using an anti-FLAG antibody directed against the FLAG epitope (Sigma, St. Louis, MO).

2.5.2. Analysis Of Regulation Of Mutant Receptors

Mutant receptors (FLAGA1ARΔ297, FLAGA1ARΔ310, FLAGA1AR-FL, FLAGA1AR-FL-Y288A) will be assayed for their ability to undergo agonist-
and antagonist-mediated regulation at several levels. Initially, saturation binding experiments in membranes from cells treated with the A₁AR selective agonist, N⁶-cyclopentyladenosine (CPA) and the adenosine receptor antagonist 8-para-sulfophenyltheophylline (8-pSPT) will be used to ascertain whether the density or affinity of the mutant receptors are regulated. Agonist-induced inhibition of forskolin-stimulated adenylyl cyclase will be evaluated to determine whether truncation of A₁AR or site-directed mutagenesis of tyrosine 288 to alanine affects the functional coupling of the receptor. It will also be critical to evaluate potential alterations in sequestration of different mutant receptors compared to wild type.