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Regulation of Two Subfamilies of Adenylyl Cyclase by G_i-coupled Receptors: A Possible Role During cAMP-dependent Synaptic Plasticity in the Hippocampus

by

Mark David Nielsen

A dissertation submitted in partial fulfillment

of the requirements for the degree of

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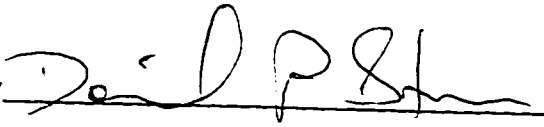
Approved by 

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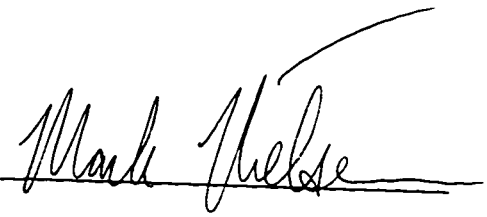
Signature 

Date June 6, 1997

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Abstract

Regulation of Two Subfamilies of Adenylyl Cyclase by G_i-coupled Receptors: A Possible Role During cAMP-dependent Synaptic Plasticity in the Hippocampus

by Mark David Nielsen

Chairperson of the Supervisory Committee

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Adenylyl cyclases catalyze the synthesis of cAMP from ATP. cAMP is an important intracellular second messenger molecule which participates in a large number of physiological processes including cellular metabolism, ion channel activity, gene transcription, and neurotransmitter release. Recently, the cAMP signal transduction pathway has also been implicated in biochemical events underlying long-term changes in synaptic strength and learning and memory. Coupling of intracellular Ca^{2+} to cAMP increases may be important for some forms of synaptic plasticity. The type 1 adenylyl cyclase (AC1) is a neural specific, Ca^{2+}-stimulated enzyme that couples intracellular Ca^{2+} to cAMP increases. Since optimal cAMP levels may be crucial for some types of synaptic plasticity, mechanisms for inhibition of Ca^{2+}-stimulated adenylyl cyclases may also be important for neuroplasticity. Here, I report that Ca^{2+} stimulation of AC1 is inhibited by activation of G_i-coupled somatostatin and dopamine D2L receptors. This inhibition is due

primarily to $G_{i\alpha}$ and not $\beta\gamma$ since coexpression of $\beta\gamma$-binding proteins with AC1 did not affect somatostatin inhibition. However, $\beta\gamma$ released from G_s did inhibit AC1, indicating that the enzyme can be inhibited by $\beta\gamma$ *in vivo* . Interestingly, the type 8 adenylyl cyclase (AC8), another Ca^{2+}-stimulated adenylyl cyclase, was not inhibited by G_i-coupled receptors. These data indicate that AC1 and AC8 are differentially regulated by G_i-coupled receptors and provide distinct mechanisms for interactions between the Ca^{2+} and cAMP signal transduction systems. I propose that AC1 may be particularly important for synaptic plasticity that depends upon rapid and transient cAMP increases, whereas AC8 may contribute to transcriptionally-dependent synaptic plasticity that is dependent upon prolonged , Ca^{2+}-stimulated cAMP increases.

Next, I sought to determine if the G_s-coupled serotonin receptors, $5HT_6$ and $5HT_7$ were capable of coupling to specific adenylyl cyclases. Serotonin is a ubiquitous neurotransmitter/hormone which elicits a variety of cellular effects via binding to a large family of plasma membrane receptors. In brain, mRNA for $5HT_6$ is found at high levels in the hippocampus, striatum, and nucleus accumbens while $5HT_7$ is expressed most abundantly in hippocampus, neocortex, and hypothalamus. Interestingly, these two serotonin receptors possess high affinity for several clinically useful antipsychotic and antidepressant drugs. To gain a better understanding of how serotonin controls cAMP levels in specific areas of brain where a specific receptor/effector coupling may exist (e.g., $5HT_6$ and AC1 in hippocampus), we coexpressed either the $5HT_6$ or $5HT_7$ receptors with specific isoforms of adenylyl cyclase in HEK 293 cells. In particular, I used the type 5 adenylyl cyclase (AC5) as a positive control, since it is sensitive to G_s-coupled receptor activation in intact cells. Additionally, I tested whether stimulation of $5HT_6$ or $5HT_7$ was able to activate AC1 or AC8. Previously, we and others have observed that AC1 is insensitive to G_s-coupled receptors unless intracellular Ca^{2+} is simulatneously elevated and CaM is bound to the enzyme. I have determined that the $5HT_6$ receptor operates as a typical G_s-coupled receptor, similar to β-adrenergic receptors, in HEK 293 cells in that it

stimulated AC5 but not AC1 or AC8. To our surprise, we observed that 5HT$_7$ not only stimulated AC5, but also AC1 and AC8. Stimulation of AC1 and AC8 occurred primarily through 5HT$_7$-mediated increases of intracellular Ca^{2+}. These results not only shed light on serotonergic control of cAMP levels in specific areas of brain, but also demonstrate that 5HT$_7$ receptors are capable of stimulating Ca^{2+}/CaM-sensitive adenylyl cyclases via increases of intracellular Ca^{2+}.

In most tissues, adenylyl cyclase is stimulated by hormones which bind to G_s-coupled receptors and is inhibited by G_i-linked hormone receptors. Paradoxically, in lung and brain, activation of G_i-coupled receptors potentiates G_s-stimulated cAMP levels. In particular, in the rodent hippocampus, an area of brain widely studied because of its role in learning and memory, activation of inhibitory hormone receptors potentiates β-adrenergic receptor stimulation of adenylyl cyclase. Interestingly, of the nine cloned mammalian adenylyl cyclases, the type 2 (AC2) and type 4 (AC4) isoforms demonstrate regulatory properties consistent with a role in G_i-mediated potentiation of G_s-stimulated cAMP levels. Since robust increases of cAMP may be involved in mechanisms underlying certain forms of synaptic plasticity, it is was important to determine the regulatory properties of AC4 in intact cells (the regulatory properties of AC2 in intact cells has already been defined), as well as determining the immunohistochemical localization of AC2 and AC4 in the hippocampus. Our results demonstrate that G_s-coupled receptor stimulation of AC4, like that for AC2, was potentiated by activation of G_i-coupled receptors *in vivo*. Additionally, we found that both AC2 and AC4 are expressed in the mouse hippocampus, especially in stratum radiatum dendrites in CA1. These observations suggest that AC2 and/or AC4 may play a crucial role in certain forms of synaptic plasticity by detecting temporally overlapping G_s and G_i synaptic inputs.

A recent observation made by Tzavara *et al* demonstrating the existence of a circadian oscillation of the AC1 mRNA in rat pineal gland has generated interest in the role of AC1 in pineal function. The pineal gland is the site of nighttime melatonin synthesis in a

variety of organisms, including mammals. Melatonin feeds back to regulate, or entrain, the endogenous circadian clock in the suprachiasmatic nucleus of the hypothalamus, as well as affecting the functions of target tissues on a circadian basis. Coincidentally, the retina also exhibits a circadian rhythm of melatonin synthesis, as well as expression of AC1. To probe the role of AC1 in pineal (and retinal) function, I have determined the mouse pineal Ca^{2+}/CaM-stimulated adenylyl cyclase activity undergoes a circadian oscillation in mice. The level of Ca^{2+}/CaM stimulation of pineal adenylyl cyclases increases ~200% at night relative to the day. On the other hand, Ca^{2+}/CaM-stimulated adenylyl cyclase activity in mouse eye cup preparations did not oscillate. This suggests that the mechanism for generating rhythmic melatonin synthesis may differ between pineal and retina.

To investigate the role of AC1 in circadian behavioral output, I monitored the locomotor activity of wild-type and AC1 knockout mice. I found that both strains show a robust circadian oscillation in their locomotor activity, with activity being highest during the dark phase. Upon closer examination, I observed several differences in the activity profiles between wild-type and AC1 mutant mice. First, the wild-type mice showed more activity and a shorter period of absolute inactivity than AC1 mutants during the light phase. Second, when subjected to a shift of the light/dark cycle, the wild-type mice adapted to the new rhythm, while the mutant mice remained entrained to the original light/dark cycle. These results indicate that AC1 may be important for an animal's ability to adapt to changes in day/night length. Further experimentation will be necessary in order to elucidate the connection, if any, between AC1, melatonin synthesis, and circadian behaviors (locomotor activity).

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LIST OF ABBREVIATIONS

AA-NAT	arylalkylamine-N-acetyltransferase
AC1	Type 1 adenylyl cyclase
AC2	Type 2 adenylyl cyclase
AC3	Type 3 adenylyl cyclase
AC4	Type 4 adenylyl cyclase
AC5	Type 5 adenylyl cyclase
AC6	Type 6 adenylyl cyclase
AC7	Type 7 adenylyl cyclase
AC8	Type 8 adenylyl cyclase
AC9	Type 9 adenylyl cyclase
ATP	adenosine triphosphate
BAPTA-AM	1,2-bis(o-amino-5,5'-difluorophenoxy)-ethane-N,N,N,N'-tetraacetic acid tetra(acetoxymethyl) ester
BCS	bovine calf serum
BSA	bovine serum albumin
CA1	CA1 field, hippocampus
CA2	CA2 field, hippocampus

CA3	CA3 field, hippocampus
Ca^{2+}	calcium ion
Ca^{2+}/CaM	the complex of four calcium ions bound to calmodulin
CaM	calmodulin
cAMP	adenosine 3',5'-cyclic monophosphate
CRE	cAMP response element
CREB	cAMP response element binding protein
D2L	the long form of the dopamine D2 receptor
DM	dissociation media
DTT	dithiothreitol
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
EGTA	[ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid]
G protein	guanine nucleotide-binding protein
GDP	guanosine 5'-diphosphate
GppNHp	5'-guanylylimidodiphosphate
GTP	guanosine 5'-triphosphate
G_i	the inhibitory guanine nucleotide-binding protein

G_o	a brain-enriched guanine nucleotide-binding protein
G_s	the stimulatory guanine nucleotide-binding protein
G_t	a retinal guanine nucleotide-binding protein (transducin)
H-DMEM	HEPES-buffered Dulbecco's modified Eagle's medium
HEK 293	human embryonic kidney 293 cells
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HIOMT	hydroxyindole-O-methyltransferase
IBMX	3-isobutyl-1-methylxanthine
L/D	light/dark
LTP	long-term potentiation
L-LTP	the long-lasting form of long-term potentiation
mf-CA3	mossy fiber-CA3
mRNA	messenger RNA
NE	norepinephrine
PBST	phosphate-buffered saline with 0.025% Triton X-100
PKA	the cAMP-dependent protein kinase
PDE	cyclic nucleotide phosphodiesterase
PKC	the Ca^{2+}/phospholipid-dependent protein kinase

PMSF	phenylmethylsulfonyl fluoride
RSV	Rous sarcoma virus
SCN	the suprachiasmatic nucleus of the hypothalamus
SDS	sodium dodecyl sulfate
TBST	Tris-buffered saline with 0.05% Tween 20
293-D2L	HEK 293 cells expressing the long form of the dopamine D2L receptor
5'-AMP	adenosine 5'-monophosphate
5HT	5-hydroxytryptamine; serotonin
α_i	the α subunit of the inhibitory guanine nucleotide-binding protein
α_s	the α subunit of the stimulatory guanine nucleotide-binding protein
α_s-GTP	the GTP-bound form of α_s
βARK$_1$-ct	the carboxy-terminal, $\beta\gamma$-binding region of β-adrenergic receptor kinase 1
$\beta\gamma$	the $\beta\gamma$ complex of heterotrimeric G proteins

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Dedication

Dedicated to my grandmother Rose Rubin. I wish you were here to share in this moment. Always remember, "All Cats are Grey".

CHAPTER I. INTRODUCTION

General Introduction

Adenylyl cyclases (EC 4.6.1.1) catalyze the synthesis of 3',5'-cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP is an important intracellular second messenger molecule which regulates a variety of physiological processes including cellular metabolism, ion channel activity, neurotransmitter release, and gene transcription. The majority of cAMP's actions are mediated by the cAMP-dependent protein kinase (PKA) (Nairn et al., 1985), although there exist classes of ion channels which are directly gated by cAMP (Kaupp, 1995; Zimmerman, 1995). cAMP is degraded to 5'-AMP by a large family of cyclic nucleotide phosphodiesterase enzymes (Beavo, 1995).

cAMP was discovered in 1957 during an investigation of liver phosphorylase activation by epinephrine and glucagon (Rall et al., 1957). The cAMP-forming enzyme adenylyl cyclase was subsequently discovered by in 1962 (Sutherland et al., 1962). Since the initial discovery of this enzyme activity, the efforts of many researchers have demonstrated the complexity of this enzyme system. In recent years, nine distinct isoforms of adenylyl cyclase have been cloned (designated AC1-AC9) (Bakalyar and Reed, 1990; Cali et al., 1994; Feinstein et al., 1991; Gao and Gilman, 1991; Ishikawa et al., 1992; Katsushika et al., 1992; Krupinski et al., 1989; Premont et al., 1992; Premont et al., 1996; Watson et al., 1994; Yoshimura and Cooper, 1992), each with a unique tissue distribution (Table 1) and pattern of regulation (Table 2) (Choi et al., 1993; Cooper et al., 1995; Sunahara et al., 1996; Taussig and Gilman, 1995). The importance of adenylyl cyclases in mammalian biology was highlighted by the awarding of a Nobel Prize in Medicine in 1994

to Dr. Alfred G. Gilman and Dr. Martin Rodbell for their work on the adenylyl cyclase system.

Adenylyl cyclase activity is regulated by numerous hormones and neurotransmitters acting on seven transmembrane domain-containing, plasma membrane receptor proteins (Gudermann et al., 1997). Receptors are coupled to adenylyl cyclase via membrane-associated, heterotrimeric guanine nucleotide-binding proteins (G proteins) (Neer, 1995). Specific G proteins are capable of positively or negatively regulating adenylyl cyclase activity (Gilman, 1984) (Figure 1). Hormones such as epinephrine and glucagon are coupled to the stimulation of adenylyl cyclase by a stimulatory G protein (G_S), while hormones such as acetylcholine and somatostatin couple to inhibition of adenylyl cyclase via an inhibitory G protein (G_i). Heterotrimeric G proteins consist of a guanine nucleotide-binding α subunit and a tightly associated $\beta\gamma$ complex. Until recently, it was thought that the α subunit determined the "phenotype" of the G protein, however, it is now appreciated that the $\beta\gamma$ complex is also capable of initiating signal transduction cascades, including regulation of adenylyl cyclases (Clapham and Neer, 1993). At this time, there are at least 20 distinct α subunits, 5 β subunits, and 10 γ subunits of G proteins (Offermans and Simon, 1996). Upon binding of a hormone to its plasma membrane receptor, the GDP-bound α subunit exchanges GTP for the bound GDP. This causes dissociation of the heterotrimer into a GTP-bound α subunit and a $\beta\gamma$ complex. Both GTP-bound α subunits and $\beta\gamma$ complexes can regulate intracellular effector molecules such as adenylyl cyclases. Therefore, adenylyl cyclase activity can be regulated positively or negatively depending on the G protein subunits and adenylyl cyclase isoform involved.

Adenylyl cyclase can also be regulated by elevations of intracellular Ca^{2+} (Choi et al., 1992). Ca^{2+}, in a complex with the ubiquitous Ca^{2+}-binding protein calmodulin (CaM; complex referred to as Ca^{2+}/CaM), can stimulate the activity of several adenylyl cyclases directly (Cali et al., 1994; Minocherhomjee et al., 1987; Wu et al., 1993), or

inhibit the activity of specific adenylyl cyclases indirectly via activation of Ca^{2+}/CaM-dependent protein kinases (Wayman et al., 1995; Wei, 1996). Ca^{2+}-stimulated adenylyl cyclase activity was originally identified in bovine brain by Brostrom and coworkers (Brostrom et al., 1975). Using CaM-sepharose affinity chromatography, Westcott *et al* successfully separated bovine brain CaM-sensitive adenylyl cyclase activity from CaM-insensitive adenylyl cyclase activity (Westcott et al., 1979). This was the first demonstration that there were at least two distinct isoforms of adenylyl cyclase. During the mid-1980's, several laboratories successfully purified CaM-sensitive adenylyl cyclase from bovine brain (Minocherhomjee et al., 1987; Pfeuffer et al., 1985; Smigel, 1986; Yeager et al., 1985). Using proteolytic fragments from a purified preparation to obtain amino acid sequence, Krupinski *et al* were able to isolate a cDNA clone for the bovine brain CaM-sensitive adenylyl cyclase (Krupinski et al., 1989). This cDNA clone was designated the type I adenylyl cyclase (AC1) and its isolation has allowed for the subsequent cloning of at least eight additional adenylyl cyclase cDNAs.

Although the physiological role of cAMP in cellular metabolism has been appreciated for some time, the functional role(s) of specific isoforms of adenylyl cyclase is largely unknown. One can postulate that a specific adenylyl cyclase is involved in a given physiological process based on the enriched expression of that adenylyl cyclase in some tissue. For example, AC3 is expressed at high levels in olfactory sensory tissue, but does this mean that it is the adenylyl cyclase mediating odorant stimulation of cAMP levels? To address the physiological functions of specific adenylyl cyclases, our laboratory has created several strains of mice which lack a specific adenylyl cyclase. Mice with the genes for AC1, or AC8 disrupted have been generated, and these mice will be extremely useful tools for probing the functions of specific adenylyl cyclases. The adenylyl cyclase field is at a crossroads where the elucidated biochemical properties of adenylyl cyclases only advances

our knowledge to some limited point. With the advent of gene disruption techniques, we can attempt to correllate the regulatory properties of adenylyl cyclases with specific behavioral phenotypes and physiological processes. These approaches will greatly enhance our understanding of the hormonal regulation of cAMP levels in specific tissues, the cellular processes in which specific adenylyl cyclases are involved, and thus, allow for an integrated approach towards intervention into specific organ systems at the cellular/molecular level as a means of therapeutic benefit. This is the direction in which the adenylyl cyclase field is heading and future results with mouse strains lacking specific adenylyl cyclases will be eagerly anticipated.

Regulation of Adenylyl Cyclases by Heterotrimeric G Proteins

A variety of hormones and neurotransmitters regulate adenylyl cyclase activity. Hormone binding to G protein-coupled plasma membrane receptors can stimulate adenylyl cyclase via G_S or inhibit adenylyl cyclase through G_i (Figure 1). Classically, β-adrenergic receptors have been shown to stimulate adenylyl cyclase via G_S. Binding of epinephrine to the β-adrenergic receptor causes GTP exchange on the α subunit of G_S followed by dissociation of the G_S heterotrimer into an α_S-GTP subunits and a βγ complex. α_S-GTP stimulates adenylyl cyclase while βγ can stimulate, inhibit, or have no effect on cAMP levels depending on the adenylyl cyclase isoform involved (Sunahara et al., 1996). When the intrinsic GTPase activity of the α subunit hydrolyzes bound GTP to GDP, the heterotrimer reassociates and signalling is terminated. Interestingly, cholera toxin ADP-ribosylates $G_{S\alpha}$ and inhibits its GTPase activity (Cassel and Pfeuffer, 1978; Gill and Meren, 1978), thereby locking $G_{S\alpha}$ in an activated state and leading to chronically elevated cAMP levels. Inhibitory receptors such as the m_2 mucarinic acetylcholine receptor or the family of somatostatin receptors inhibit adenylyl cyclase via an analagous mechanism

involving G_i. Pertussis toxin, a bacterial toxin which also chronically elevates cAMP levels, works by catalyzing the ADP-ribosylation of $G_{i/o\alpha}$ only when in the intact heterotrimer (Burns et al., 1983; Hsia et al., 1984; Katada and Ui, 1982; Katada and Ui, 1982; Neer et al., 1984). ADP-ribosylation of $G_{i/o\alpha}$ uncouples it from its cognate receptors and prevents inhibitory signalling (West et al., 1985).

In vitro, all adenylyl cyclases can be stimulated by GTP-bound $G_{s\alpha}$ (Sunahara et al., 1996; Taussig and Gilman, 1995). However, responses to both $G_{i\alpha}$ and $\beta\gamma$ vary among adenylyl cyclase isoforms. For example, AC1, AC5, and AC6 are inhibited by $G_{i\alpha}$ *in vitro* (Taussig et al., 1994), whereas AC2 and AC4 are unaffected (Sunahara et al., 1996; Taussig and Gilman, 1995; Taussig et al., 1994; Taussig et al., 1993). $\beta\gamma$ can inhibit AC1 under certain conditions (Taussig et al., 1994; Taussig et al., 1993), AC2 and AC4 are stimulated by $\beta\gamma$ (Gao and Gilman, 1991; Tang and Gilman, 1991), and AC3, AC5, AC6 are insensitive to the $\beta\gamma$ complex. Stimulation of AC2 by $\beta\gamma$ occurs (Federman et al., 1992), and stimulation is conditional upon $G_{s\alpha}$ activation of the enzyme. For AC2, protein kinase C (PKC) activation can substitute for $G_{s\alpha}$ with regard to $\beta\gamma$ stimulation (Tsu and Wong, 1996). It is not known if AC4 is stimulated by $\beta\gamma$. Ca^{2+}/CaM-stimulated AC1 is inhibited by both $G_{i\alpha}$ and $\beta\gamma$ *in vitro* (Tang et al., 1991; Tang and G., 1991; Taussig et al., 1994; Tota et al., 1990), however it is unclear if and how inhibition is accomplished upon activation of inhibitory hormone receptors. Interestingly, AC1 is not stimulated by G_s-coupled receptors unless intracellular Ca^{2+} is simultaneously elevated (Wayman et al., 1994). Is it possible that the lack of G_s stimulation of AC1 is due to inhibition by $\beta\gamma$ released from G_s?

The presence of Ca^{2+}/CaM-stimulated adenylyl cyclase in brain was originally demonstrated by Brostrom *et al* in 1975 (Brostrom et al., 1975). Westcott *et al* were the first to separate CaM-sensitive from CaM-insensitive adenylyl cyclase activity in bovine brain membrane preparations (Westcott et al., 1979). Utilizing CaM-sepharose affinity chromatography, Westcott *et al* isolated two fractions of adenylyl cyclase activity. Fraction I was CaM-insensitive and later shown to be stimulated by the non-hydrolyzable GTP analog GppNHp in the presence of solubilized brain membranes (Toscano et al., 1979), while fraction II, eluted with EGTA, was stimulated by CaM in the presence of Ca^{2+}. This was the first demonstration of the presence of at least two distinct isoforms of adenylyl cyclase in brain. Subsequent demonstrations of CaM-sensitive and CaM-insensitive adenylyl cyclase activities in brain were accomplished by generation of antibodies which distinguished between CaM-sensitive and insensitive adenylyl cyclase (Mollner and Pfeuffer, 1988; Rosenberg and Storm, 1987).

CaM-sensitive adenylyl cyclase from bovine brain has been highly purified by several laboratories (Minocherhomjee et al., 1987; Pfeuffer et al., 1985; Smigel, 1986; Yeager et al., 1985). The catalytic subunit runs between 120-150kD on SDS gels. Often times, G protein subunits copurified with CaM-sensitive adenylyl cyclase (Yeager et al., 1985), however, with the advent of forskolin-sepharose affinity chromatography (Pfeuffer et al., 1985), preparations devoid of G proteins were isolated. Interestingly, it was observed that enhanced purification of bovine brain Ca^{2+}/CaM-sensitive adenylyl cyclase could be acheived using immobilized wheat germ agglutinin (Minocherhomjee et al., 1987; Smigel, 1986), suggesting that the purified product was a glycoprotein. In fact, Minocherhomjee *et al* demonstrated that iodinated wheat germ agglutinin interacted directly with purified Ca^{2+}/CaM-sensitive adenylyl cyclase (Minocherhomjee et al., 1987). With the advent of molecular cloning techniques, Krupinski *et al* utilized tryptic peptides from

purified CaM-sensitive adenylyl cyclase to obtain amino acid sequence information and subsequently cloned the bovine brain CaM-sensitive adenylyl cyclase and designated it the type 1 adenylyl cyclase (Krupinski et al., 1989).

In 1994, a second Ca^{2+}/CaM-stimulated adenylyl cyclase was cloned from a rat brain cDNA library (Cali et al., 1994). This isoform was designated AC8 and its discovery was not completely unexpected since brains from AC1 knockout mice still contained residual Ca^{2+}/CaM-stimulated adenylyl cyclase activity (Wu et al., 1995). Interestingly, the Ca^{2+} sensitivity of the residual Ca^{2+}-stimulated adenylyl cyclase activity in AC1 mutant mice brains was shifted to the right when compared with wild-type mouse brain membrane preparations (Wu et al., 1995). The meaning of this result was borne out when the Ca^{2+} sensitivity of AC8 expressed in human embryonic kidney 293 (HEK 293) cells was found to be ~5-fold lower than that of AC1 (Villacres et al., 1995). The EC_{50} for Ca^{2+} stimulation of AC1 is ~150nM, while the EC_{50} for AC8 was ~750nM (Villacres et al., 1995). Therefore, the two Ca^{2+}/CaM-stimulated adenylyl cyclases are not redundant enzymatic activities since they differ in at least one key regulatory feature, that is, their Ca^{2+} sensitivities. Work detailed in this thesis will demonstrate further differences between AC1 and AC8.

Structure of Mammalian Adenylyl Cyclases

Mammalian adenylyl cyclases are integral membrane glycoproteins containing one or more consensus sites for N-linked glycosylation (Taussig and Gilman, 1995). Structurally, they are thought to resemble several voltage-gated ion channels and ATP-dependent transporters (Krupinski et al., 1989). Hydropathy analysis predicts the following structure for adenylyl cyclases (Figure 2): a short, variable-length cytoplasmic amino terminus (designated N), followed by six transmembrane-spanning streches

(designated M1), a large intracellular loop (designated C1), a second set of six transmembrane-spanning segments (designated (M2), and a large, cytoplasmic carboxy-terminal region (designated C2). Regions of the large cytoplasmic domains of adenylyl cyclases share significant homology with each other as well as with the catalytic domains of soluble guanylyl cyclases (Taussig and Gilman, 1995). It is thought that the cytoplasmic domains of adenylyl cyclases function in enzyme catalysis. In fact, chimeric adenylyl cyclase molecules in which the two cytoplasmic domains are covalently attached by a linker peptide remain catalytically active (Tang and Gilman, 1995; Yan et al., 1996). Additionally, non-covalent chimeras in which the two cytoplasmic domains are simply coexpressed also show catalytic activity (Dessauer and Gilman, 1996; Whisnant et al., 1996). Zhang *et al* have obtained the crystal structure of dimers of the second cytoplasmic domain of AC2. In addition to delineating the forskolin- and ATP-binding pockets, their data suggest that forskolin enhances the interaction between adjacent catalytic regions, possibly by facilitating or stabilizing ATP binding (Zhang et al., 1997). Similar observations have been made with a soluble, chimeric adenylyl cyclase (Whisnant et al., 1996). Therefore, emerging evidence suggests that the two halves of adenylyl cyclase interact during catalysis and that activators of the enzyme may enhance or facilitate this interaction. The effect of inhibitors such as $G_{i\alpha}$ on this catalytic interation has not been determined.

Recent work from our laboratory has determined that one or more isoforms of adenylyl cyclase may be sensitive to changes in membrane potential. Reddy *et al* have detected a voltage-sensitive adenylyl cyclase activity in cultured cerebellar neurons (Reddy et al., 1995). Although the mechanism of voltage-sensitivity of adenylyl cyclase is unclear, the structural resemblance between adenylyl cyclases and voltage-gated ion channels may provide some clues. Ion channels contain a series of charged amino acid residues in specific transmembrane segments of the voltage-sensitive subunit of the protein, and these

amino acid residues serve as a voltge-sensor. (Catterall, 1995; Hille, 1992). Several adenylyl cyclases also have charged amino acids in the membrane-spanning segments, thus providing a possible mechanism by which changes in membrane potential can alter the conformation of adenylyl cyclases and render the adenylyl cyclase more sensitive to specific activators such as G_S (Reddy et al., 1995). Future studies will be required to fully elucidate the mechanism for voltage-sensitive changes in adenylyl cyclase activity.

Tissue Distribution of Adenylyl Cyclase Isoforms

Historically, the lack of subtype specific antibodies to adenylyl cyclases has prevented a detailed cellular and tissue distribution of individual adenylyl cyclase isozymes at the protein level. However, mRNA disribution patterns for most of the adenylyl cyclases have been published (Table I). AC1 is a neural-specific protein expressed only in brain, retina, and adrenal medulla, each a neurally-derived tissue (Xia et al., 1993). AC2 is mainly expressed in brain and lung, although AC2 mRNA expression is also reported to occur in skeletal muscle (Torgan and Kraus, 1996). AC3, originally thought to be specific to olfactory tissue, has a broad tissue distribution including brain, heart, adrenal, and lung (Xia et al., 1992). AC4 mRNA is expressed in brain (Gao and Gilman, 1991)and lung (Pian and Dobbs, 1995), yet it is also detected in a variety of tissues by reverse transcriptase-polymerase chain reaction strategies (Gao and Gilman, 1991). AC5 is also broadly distributed, with highest levels in cardiac tissue (Ishikawa and Homcy, 1997) and specific areas of brain (Glatt and Snyder, 1993; Mons and Cooper, 1994). AC6 mRNA is also broadly distributed at low levels (Katsushika et al., 1992), with weak expression in brain (Mons and Cooper, 1994). AC7 mRNA is detected in brain, spleen, heart, and lung (Watson et al., 1994). A second group has cloned AC7 from bovine retinal pigment epithelium (Volkel et al., 1996), suggesting a role for AC7 in the visual system. AC8

expression has not been systematically characterized, however its mRNA is expressed in brain (Cali et al., 1994) and the residual Ca^{2+}/CaM-stimulated adenylyl cyclase activity from brains of AC1 mutant mice resembles AC8 (Villacres et al., 1995; Wu et al., 1995). AC9 appears to be widely distributed both in brain and non-neuronal tissues (e.g., pancreas, kidney, liver, skeletal muscle) (Premont et al., 1996). In fact, AC9 may be the broadly distributed adenylyl cyclase associated with a metabolic role for cAMP.

In brain, AC1 is expressed in regions associated with synaptic plasticity including the hippocampus, cortex, and cerebellum (Xia et al., 1991). Specifically, AC1 mRNA levels are highest in cerebellar granule cells, the dentate gyrus, and the CA2 subfield of the hippocampus. Moderate to strong labelling was found in the CA3 and CA1 subfields of the hippocampus, neocortex, and several thalamic nuclei. In addition, recent evidence has shown AC1 mRNA to undergo a circadian oscillation in rat pineal gland (Tzavara et al., 1996), the site of nocturnal melatonin synthesis in mammals. Finally, AC1 mRNA is detected in several cell types in retina (Xia et al., 1993). AC2 mRNA is expressed in several areas of brain as well. Highest expression levels are found in the dentate gyrus and CA1 region of the hippocampus, olfactory bulb, piriform cortex, dorsal raphe, locus coeruleus, as well as several thalamic and hypothalamic nuclei. Moderate labelling is detected in large striatal neurons, cerebellar Purkinje cells, neocortex, and the suprachiasmatic nucleus of the hypothalamus (Furuyama et al., 1993). AC3 is expressed mainly in the olfactory bulb, however moderate mRNA signals can be found in the hippocampus and cerebellum (Glatt and Snyder, 1993). There is no information concerning AC4 mRNA distribution in brain. AC5 appears to be expressed selectively in dopaminergically-innervated brain regions, mainly striatum and nucleus accumbens (Glatt and Snyder, 1993; Mons and Cooper, 1994). The expression pattern of AC6 in brain has not been well-characterized, but evidence to date suggests that levels are quite low throughout brain (Mons and Cooper, 1994). AC7 is mainly expressed in cerebellar granule

cells, however, even in that region expression is quite low compared to AC1 and AC2 (Hellevuo et al., 1995). Highest levels of AC8 mRNA are found in hippocampus, entorhinal cortex, neocortex, hypothalamus, and amygdala (Cali et al., 1994). AC9 is found in hippocampus, neocortex, cerebellum, striatum, and amygdala (Premont et al., 1996). Thus, it appears as though all of the cloned mammalian adenylyl cyclases are expressed in brain to varying degrees. It is likely that both their differential regulation and expression patterns contribute to the functional roles of each adenylyl cyclase in brain. However, for the most part physiological functions of specific adenylyl cyclases has yet to be determined.

Role of Adenylyl Cyclases in Synaptic Plasticity

Although it has traditionally been difficult to identify the physiological functions of specific adenylyl cyclases, mounting evidence suggests that the Ca^{2+}/CaM-stimulated adenylyl cyclases play a role in synaptic plasticity (Xia et al., 1995). Loosely defined, synaptic plasticity refers to the ability of neurons to exhibit long-term changes in their responsiveness to specific stimulus patterns, and such changes are thought to be neural substrates for learning and memory (Bliss and Collingridge, 1993; Chen and Tonegawa, 1997). Evidence supporting a role for Ca^{2+}/CaM-stimulated adenylyl cyclases in synaptic plasticity comes from a variety of sources. First, studies of associative learning in the marine mollusk *Aplysia* suggest that a Ca^{2+}/CaM-stimulated adenylyl cyclase is important for the occurrence of the associative learning (Abrams et al., 1991). Similar results have also been observed in the *Drosophila* associative learning mutant *rutabaga*, which specifically lacks a Ca^{2+}/CaM-stimulated adenylyl cyclase (Livingston et al., 1984). The gene for this enzyme maps to the rut locus in the *Drosophila* genome (Levin et al., 1992). In rat brain, AC1 expression is neural-specific (Xia et al., 1993) and predominantly found

in areas of the brain associated with learning and memory such as the hippocampus, neocortex, and cerebellum (Xia et al., 1991). Mutant mice lacking AC1 exhibit alterations in several forms of long-term potentiation (LTP), a cellular model for synaptic plasticity (Bliss and Collingridge, 1993; Chen and Tonegawa, 1997), as well as deficits in spatial memory. Specifically, AC1 knockout mice show alterations in the initial, rising phase of the long-lasting form of LTP (L-LTP) (Wu et al., 1995) and severely compromised LTP at the mossy fiber-CA3 synapse (mf-CA3) (Villacres and Storm, unpublished observations). AC1 mutant mice also seem to have deficits in spatial memory as determined in the transfer test of the Morris water task (Morris et al., 1986; Morris et al., 1982; Wu et al., 1995). Finally, it was recently demonstrated that mf-CA3 LTP is dependent on cAMP (Huang et al., 1995; Huang et al., 1994; Weisskopf et al., 1994), leading to the hypothesis that Ca^{2+} coupling to increases in cAMP synthesis in mossy fiber terminals results in a persistent enhancement of glutamate release thus eliciting LTP at that synapse.

Regarding synaptic plasticity, it has been proposed that during L-LTP in the CA1 elevations of intracellular Ca^{2+} trigger activation of the Ca^{2+}/CaM-stimulated adenylyl cyclases and subsequent activation of PKA. PKA is then able to phosphorylate and activate the cAMP-response element binding protein (CREB) transcription factor which stimulates transcription of cAMP-responsive genes thought to be important for long-term changes in neuronal behavior. Interestingly, in the CA1 region of hippocampus where the induction of L-LTP may be postsynaptic in origin, L-LTP depends on PKA (Frey et al., 1993; Matthies et al., 1990) and new gene transcription (Nguyen et al., 1994). The stimulus paradigm which generates L-LTP in CA1 also induces cAMP-responsive transcription (Impey, 1996). At the mf-CA3 synapse where LTP originates presynaptically, Weisskopf *et al* demonstrated that this form of synaptic plasticity was dependent on cAMP (Weisskopf et al., 1994). These authors suggest that the initial, tetanus-induced Ca^{2+} elevations are coupled to cAMP synthesis through one of the

Ca^{2+}/CaM-stimulated adenylyl cyclases. Specifically, Ca^{2+} elevations increase cAMP synthesis, causing activation of PKA followed by an increase in glutamate release by an undetermined mechanism.

Evidence for participation of CaM-insensitive adenylyl cyclases in synaptic plasticity is limited. At specific synapses in the hippocampus, several laboratories have observed G_i-mediated potentiation of G_s-stimulated cAMP levels (Andrade, 1993; Gereau IV and Conn, 1994). Since robust increases in cAMP levels may be required for certain forms of synaptic plasticity (Backsai et al., 1993; Hagiwara et al., 1993), an adenylyl cyclase such as AC2 or AC4, which respond to paired G_s and G_i inputs by showing "suprastimulation", would be an attractive candidate to play a role in long-term changes in synaptic strength. AC2 mRNA has been detected in the dentate gyrus and CA1, however, no information regarding brain localization of AC4 is currently available. Interestingly, several groups have demonstrated that pertussis toxin prevents the development of LTP in CA3 (Ito et al., 1988) and CA1 (Goh and Pennefather, 1990; Goh and Pennefather, 1989). Therefore, a role for G_i in certain forms of LTP is plausible. Consistent with a role for pertussis toxin-sensitive G proteins during LTP, activation of G_i/G_o-coupled opioid receptors is necessary for LTP at the mf-CA3 synapse (Williams and Johnston, 1996). Work detailed in this thesis will address a possible role for $\beta\gamma$-stimulated adenylyl cyclases in synaptic plasticity.

Role of Adenylyl Cyclases in Circadian Rhythms

An additional physiological role for AC1 in the control of the circadian rhythm of melatonin synthesis in pineal has been suggested. Tzavara *et al* demonstrated that in pineal, AC1 mRNA undergoes a circadian oscillation with levels being highest during the daytime

and lowest at night (Tzavara et al., 1996). Consequently, pineal Ca^{2+}/ CaM-stimulated adenylyl cyclase activity is highest at night. In pineal, melatonin is synthesized only at night when norepinephrine (NE) release from superior cervical ganglion fibers innervating the pineal increases cAMP levels and stimulates transcriptional activation of the arylalkylamine N-acetyltransferase (AA-NAT) gene (Baler et al., 1997; Borjigin et al., 1995; Roseboom et al., 1996; Roseboom and Klein, 1995). AA-NAT is the rate-limiting enzyme in melatonin biosynthesis from serotonin (Klein, 1985; Klein and Weller, 1970). NE binds to both α_1 and β-adrenergic receptors in pineal, thus, eliciting increases in intracellular Ca^{2+} and activation of G_S. Interestingly, AC1 is synergistically stimulated by Ca^{2+}/CaM and G_S-coupled receptors (Impey et al., 1994; Wayman et al., 1994). Since robust increases in cAMP levels may be required for PKA phosphorylation and subsequent activation of CREB (Hagiwara et al., 1993), the regulatory properties of AC1 appear uniquely suited to drive activation of cAMP-responsive enhancer elements in the promoter of the AA-NAT gene in pineal (Baler et al., 1997). Therefore, both the regulatory properties of AC1 as well as its circadian fluctuation in rat pineal support a role for AC1 in maintaining the circadian oscillation in melatonin biosynthesis.

Figure 1. Regulation of adenylyl cyclases by G protein-coupled receptors and Ca^{2+}/CaM. Hormone binding to plasma membrane receptor proteins (Rs or Ri) regulates the activity of adenylyl cyclase via heterotrimeric guanine nucleotide-binding proteins. In general, the α subunit of the G protein conveys a stimulatory (α_s) or an inhibitory (α_i) signal form the receptor to the adenylyl cyclase. Additionally, elevations of intracellular Ca^{2+} can stimulate the activity of several adenylyl cyclase isoforms. The cellular effects of increased cAMP levels are primarily mediated by PKA. cAMP is degraded to 5'-AMP by cyclic nucleotide phosphodiesterases.

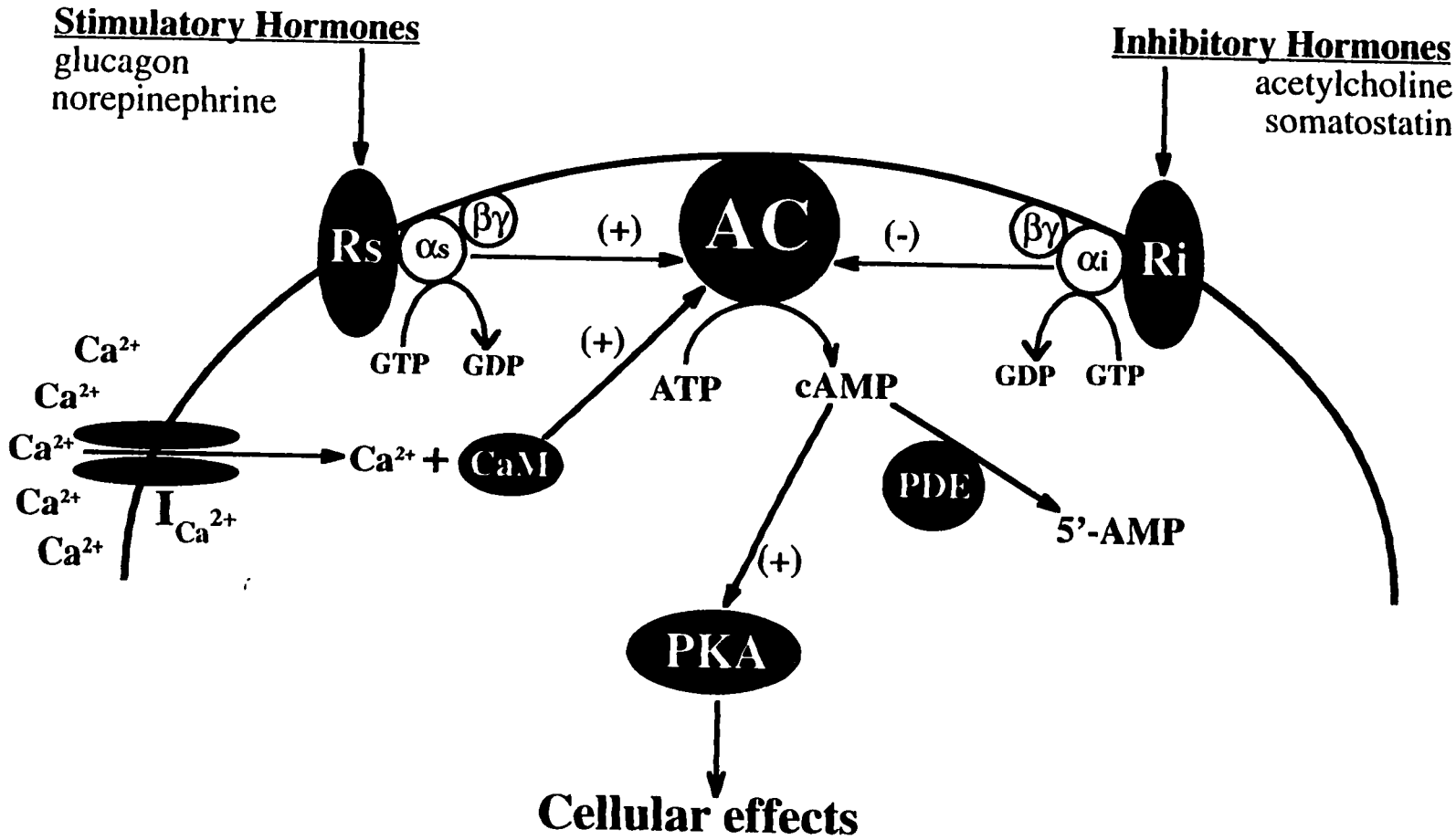

Stimulatory Hormones
glucagon
norepinephrine
Inhibitory Hormones
acetylcholine
somatostatin
Rs
αs
βγ
(+)
AC
(-)
βγ
αi
Ri
GTP
GDP
GDP
GTP
(+)
ATP
cAMP
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
ICa2+
Ca2+ + CaM
PDE
5'-AMP
(+)
PKA
Cellular effects

Figure 2. Predicted membrane topology of mammalian adenylyl cyclases. From hydropathy analysis, adenylyl cyclases are predicted to have the following structure : a short, variable-length, cytoplasmic NH_2-terminal stretch (N) followed by six transmembrane-spanning segments (M_1), a large cytoplasmic loopdivided into two regions (C_{1a} and C_{1b}), a second set of six transmembrane-spanning segments (M_2), and a variable-length cytoplasmic COOH-terminal tail divided into two parts (C_{2a} and C_{2b}). C_{1a} and C_{2a} (bold lines) share significant homology with each other as well as with the catalytic domains of soluble guanylyl cyclases. The extracellular loop between transmembrane domains 9 and 10 contains a consensus site for N-linked glycosylation. Figure reproduced from Taussig, R. and Gilman, A.G., 1995.

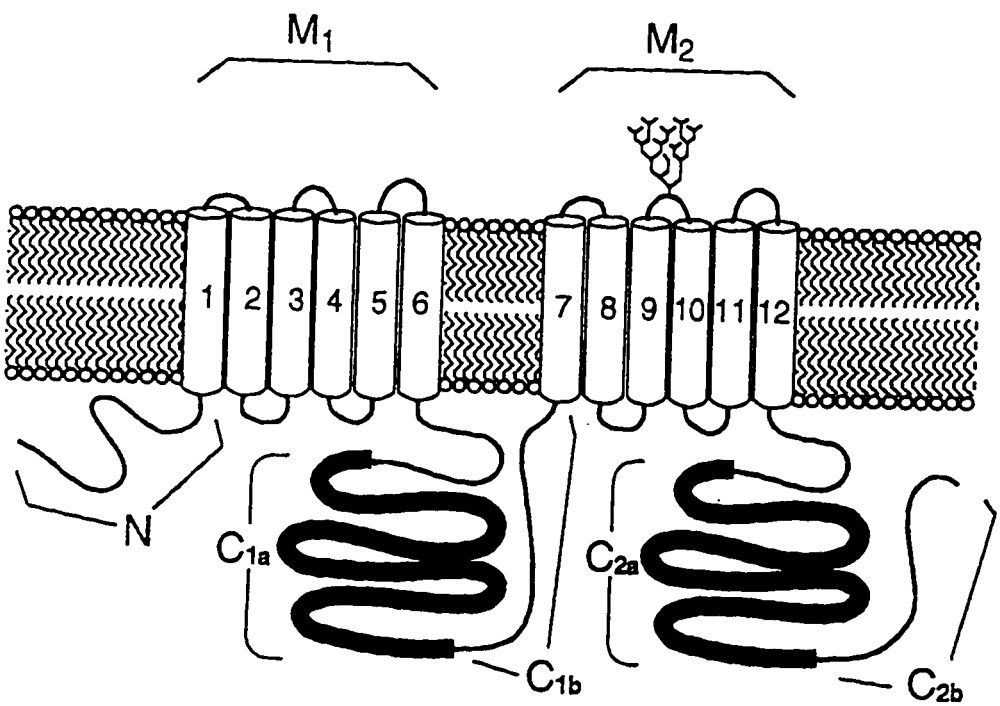

M1
M2
1
2
3
4
5
6
7
8
9
10
11
12
N
C1a
C1b
C2a
C2b

Table I

Tissue Distribution of the Mammalian Adenylyl Cyclases

Type	mRNA (kb)	Mr (kD)	mRNA Distribution
1	11.5	120	brain, retina, adrenal medulla (neurospecific)
2	4.1	119	brain, lung, olfactory epithelium, olfactory bulb, skeletal muscle
3	4.7	125	olfactory epithelium, brain, heart, adrenal, lung, retina, adipose
4	3.5	117	brain, lung, heart, liver, kidney
5	5, 7	119	heart, brain, kidney, lung, liver, adrenal, gut
6	6.1	132	heart, brain, intestine, lung, spleen
7	7.25	120	lung, spleen, brain, heart
8	4.4, 5.5	125	brain (an extensive survey has not been reported)
9	8.5	140	brain, skeletal muscle, heart, lung, liver, kidney

Table II

Regulatory Properties of the Mammalian Adenylyl Cyclases *in vivo* (ca. 1995)

Type	Gs-coupled R	Gi-coupled R	Ca2+	βγ
1	insensitive	ND	stimulated	ND
2	stimulated	stimulated	insensitive	stimulated
3	stimulated	ND	inhibited	ND
4	ND	ND	ND	ND
5	stimulated	ND	inhibited	ND
6	stimulated	ND	inhibited	ND
7	stimulated	ND	insensitive	ND
8	insensitive	ND	stimulated	ND

ND - not determined

CHAPTER II. DIFFERENTIAL REGULATION OF THE TYPE 1 AND TYPE 8 CALCIUM/CALMODULIN-STIMULATED ADENYLYL CYCLASES BY Gi-COUPLED RECEPTORS *IN VIVO*

Introduction

Cross-talk between the Ca^{2+} and cyclic AMP signal transduction systems is one of several mechanisms important for synaptic plasticity (Abrams et al., 1991; Choi et al., 1993; Feany, 1990; Levin et al., 1992; Livingston et al., 1984; Weisskopf et al., 1994; Wu et al., 1995; Xia et al., 1995). There are multiple mechanisms for Ca^{2+} regulation of adenylyl cyclases, including activation by protein kinase C (types 1, 2, 3, 5, 7) (Choi et al., 1993; Jacobowitz and Iyengar, 1994; Kawabe et al., 1994; Lustig et al., 1993; Watson et al., 1994; Yoshimura and Cooper, 1993), stimulation by calmodulin (CaM) and Ca^{2+} (types 1 and 8) (Cali et al., 1994; Choi et al., 1993; Cooper et al., 1995; Tang and Gilman, 1992; Taussig and Gilman, 1995), inhibition by CaM-dependent protein kinase II (type 3) (Wayman et al., 1995; Wei, 1996) and CaM-dependent protein kinase IV (type 1) (Wayman et al., 1996), as well as direct inhibition by Ca^{2+} (types 5 and 6) (Ishikawa et al., 1992; Katsushika et al., 1992; Mons and Cooper, 1994; Yoshimura and Cooper, 1992). The Ca^{2+}/CaM-stimulated adenylyl cyclases, type I (AC1) and type VIII (AC8) adenylyl cyclases (Cali et al., 1994; Krupinski et al., 1989; Tang et al., 1991; Xia et al., 1993), are expressed in areas of the brain associated with learning and memory including the hippocampal formation (Cali et al., 1994; Xia et al., 1991) and mice lacking AC1 have deficits in spatial memory and altered long-term potentiation (LTP) (Wu et al., 1995).

Mechanisms for attenuation of cAMP signals generated by the Ca^{2+}-stimulated adenylyl cyclases *in vivo* have not been defined. Inhibition of the Ca^{2+}/CaM-stimulated adenylyl cyclases may be important during synaptic plasticity since optimal, not necessarily

maximal, cAMP levels are necessary for certain forms of learning (Feany, 1990). Specifically, the sensitivity of Ca^{2+}-stimulated AC1 or AC8 to G_i-coupled receptor activation has not been reported. Although AC1 is inhibited by $G_{i\alpha}$ *in vitro* (Taussig et al., 1993; Taussig et al., 1994), regulatory mechanisms determined with purified enzymes or membrane preparations are not necessarily operative *in vivo*. For example, AC1 is stimulated by activated $G_{s\alpha}$ in membranes, however it is not stimulated by G_s-coupled receptor activation *in vivo* (Wayman et al., 1994). Additionally, G_s activation of AC3 is potentiated by Ca^{2+}/CaM *in vitro* (Choi et al., 1992), however *in vivo*, elevations of intracellular Ca^{2+} inhibit G_s stimulation of AC3 (Wayman et al., 1995; Wei, 1996). Therefore, it was important to determine the responses of AC1 and AC8 to G_i-coupled receptors in intact cells. In this study, we report that AC1, but not AC8, is inhibited by activation of somatostatin or dopamine D2L receptors in HEK 293 cells. A comparison of AC1 with AC8 indicates that these two Ca^{2+}-stimulated enzymes have distinct regulatory properties.

Materials and Methods

Materials- 3-isobutyl-1-methylxanthine (IBMX), carbamylcholine chloride (carbachol), dopamine, forskolin, isoproterenol, serotonin, and somatostatin-14 were from Sigma. A23187 was purchased from Calbiochem. Pertussis toxin was obtained from List Biological (Campbell, CA).

Cell Culture- Human embryonic kidney 293 (HEK 293) cells were grown at 37°C in HEPES-buffered Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 95% air/5% CO_2 incubator. HEK 293 cells

stably expressing the long form of the dopamine D2 receptor (293-D2L) were generously provided by Dr. Kim Neve (VA Medical Center, Portland, OR). 293-D2L cells require maintenance in 2μg/ml puromycin (Aldrich). Cell culture materials were from Life Technologies, Inc. unless otherwise indicated.

Expression of AC1 and AC8 in HEK 293 Cells- The AC1 cDNA clone was isolated from a bovine brain cDNA library as described (Xia et al., 1991). The cDNA clone for AC8 was generously provided by Dr. John Krupinski (Weis Center for Research, Geisinger Clinic, Danville, PA). Polyclonal populations of hygromycin (Calbiochem, 500units/ml)-resistant 293 cells or 293-D2L cells were obtained by stable transfection of the pCEP4 expression vector (Invitrogen), pCEP4-AC1, or pCEP4-AC8 by the calcium phosphate method (Chen and Okayama, 1987). All stable cell lines were created from the same parental population of HEK 293 cells or 293-D2L cells. Expression of transfected adenylyl cyclases was determined by cAMP accumulation assays as described below.

cAMP Accumulation Assay- Changes in intracellular cAMP levels were measured by determining the ratio of [^{3}H]-cAMP to a total ATP, ADP, and AMP pool in [^{3}H]-adenine-loaded cells as described (Wong et al., 1991). This assay system allows for rapid and sensitive determination of relative changes in intracellular cAMP levels. While the ratios measured between assays may show some variation, the relative changes in cAMP levels between assays is quite reproducible. Briefly, as cells in six-well plates approached confluency (~90%), they were incubated in H-DMEM+10% fetal bovine serum containing 2μCi/ml [^{3}H]-adenine for 16-20 hours. The next day, cells were aspirated, washed once with 150mM NaCl and incubated in buffer A (20mM HEPES pH 7.4, 120mM NaCl, 5mM KCl, 2.5mM $MgSO_4$, 2mM $CaCl_2$, 10mM glucose, 2mM sodium phosphate) or H-

DMEM+1% pen/strep containing the indicated effectors (e.g., A23187, carbachol, dopamine, forskolin, isoproterenol, serotonin, somatostatin) plus 1mM IBMX. For extracellular Ca^{2+} dose-responses, Buffer A was made with varying concentrations of $CaCl_2$. Reactions were terminated by aspiration and addition of 1 ml ice-cold 5% trichloroacetic acid/1μM cAMP. Culture dishes were maintained at 4°C for 1-4 hours and acid-soluble nucleotides were separated by sequential Dowex AG50WX-4 and neutral alumina chromatography as described (Salomon et al., 1974). Reported data are the averages of triplicate determinations. Pertussis toxin (List Biological), when used, was added to cells along with [^{3}H]-adenine for 16-20 hours.

Transient Coexpression of AC1 with the Carboxy-terminus of β-Adrenergic Receptor Kinase 1 or Transducin-α in HEK 293 cells-The peptide minigene construct encoding the carboxy-terminus of β-adrenergic receptor kinase 1 (βARK1-ct) in the pRK5 plasmid (Koch et al., 1994) was generously provided by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). A cDNA clone for the α subunit of human rod transducin was provided by Dr. Neil M. Nathanson (University of Washington, Seattle, WA). The G_s-coupled serotonin receptor 5HT7 cDNA (Shen et al., 1993) was a gift from Dr. Mark Hamblin (VA Medical Center, Seattle, WA). Briefly, the night before transfection, cells were plated in 100mm plates at 70% density. The following morning, each plate was transfected with 8μg total DNA (1μg 5HT7, 2.5μg pCEP4, pCEP4-AC1, or pCEP4-AC4, 4μg pcDNAIII (Invitrogen), pcDNAIII-transducin-α, or pRK-βARK1-ct, and 0.5μg RSV-β-galactosidase) in H-DMEM in the presence of 50-60μl of Lipofectamine (Life Technologies, Inc.). After 5 hours, cells were rinsed with H-DMEM+1% pen/strep+10% FBS and maintained for 24 hours under normal conditions. The following day, cells were split, pooled by transfection, and seeded into 6-well culture dishes (1 transfected plate/6-well dish) for cAMP assays as well as into 12-well plates (2 wells/transfection) for β-

galactosidase assays. The next morning, cells used for cAMP assays were labelled for 4-6 hours with 2-3μCi/well [^{3}H]-adenine (ICN). Just prior to the cAMP assay, companion cells for β-galactosidase assays were lysed in 500μl of buffer B (100mM KH_2PO_4 pH 7.8, 0.2% Triton X-100, 1mM DTT) and frozen until use. cAMP and β-galactosidase assays were carried out as described and all raw data was normalized to the measured β-galactosidase signal for each transfection.

β–Galactosidase Assay- Lysates of transiently transfected cells (described above) were thawed and centrifuged at 16,000x*g* for 10 minutes. The supernatant (20μl) was combined with 100μl of reaction buffer (100mM Na_2HPO_4 pH 8.0, 1mM $MgCl_2$, 35mM Galacton (Tropix, Bedford, MA), 100mM D-galactose) and incubated in the dark at room temperature for 60 minutes. During this incubation period, a 10% solution of Emerald (Tropix, Bedford, MA) in 0.2N NaOH was prepared for subsequent addition to the samples at 5 second intervals by a Berthold luminometer. Each well of lysed cells was assayed in duplicate and data was used to normalize for transfection efficiency.

Primary Neuron Cultures- Primary cortical and hippocampal neurons were cultured essentially as described in Impey *et al* (Impey et al., 1994) and maintained in NeuroBasal media supplemented with B27. Briefly, dissected brain regions were minced in Dissociation Media (DM) (75mM Na_2SO_4, 3.3mM K_2SO_4, 15mM $MgCl_2$, 0.225mM $CaCl_2$, 18mM glucose, 4mM kynurenic acid, 2.0mM HEPES pH 7.4) and incubated in DM containing 100U/ml activated papain (Worthington) with gentle agitation at 37°C for 30 minutes. The disrupted tissue was then rinsed with DM and cells were dissociated by gentle trituration through a 2 ml serological pipette. Cells were plated at a density of $5x10^5$ cells/well in 12-well culture dishes coated with poly-D-lysine (50-66μg/mL).

Results

Inhibition of Ca^{2+} Stimulated AC1 by G_i-Coupled Receptors in vivo- To examine the effect of G_i-coupled receptors on AC1 *in vivo*, we carried out an initial screen of HEK 293 cells to determine if they express endogenous inhibitory receptors that couple to adenylyl cyclases. cAMP levels were elevated 6-fold by activation of endogenous β-adrenergic receptors with isoproterenol (Wayman et al., 1994). Application of 1μM somatostatin inhibited isoproterenol stimulation of the endogenous adenylyl cyclase(s) by approximately 50% indicating the presence of somatostatin receptors (data not shown). Somatostatin receptors are seven transmembrane domain proteins which typically couple to the G_i/G_o class of heterotrimeric G proteins (Bell and Reisine, 1993; Reisine and Bell, 1995).

HEK 293 cells do not express Ca^{2+}-stimulated adenylyl cyclases and treatment with the Ca^{2+} ionophore A23187 or the muscarinic receptor agonist carbachol does not elevate intracellular cAMP (Choi et al., 1992). However, carbachol or A23187 activates AC1 expressed in HEK 293 cells by increasing intracellular Ca^{2+} (Choi et al., 1992). A23187 elevated intracellular cAMP levels approximately 25-fold and somatostatin inhibited Ca^{2+}-stimulated activity with an IC_{50} of approximately 10nM (Figure 3A). Carbachol stimulation of AC1 was also inhibited approximately 50% by 1μM somatostatin (data not shown, but see Figure 5A). Somatostatin inhibition of AC1 was not limited to Ca^{2+}-stimulated activities; forskolin-stimulated AC1 was inhibited 41% by 500 nM somatostatin (data not shown, but see Figure 12B). To determine if other G_i-coupled receptors also couple to inhibition of AC1, HEK 293 cells expressing the dopamine D2L receptor were stably transfected with AC1. In these cells, dopamine maximally inhibited AC1 activity 75% with an IC_{50} of 50 nM (Figure 3B).

The effect of somatostatin on Ca^{2+}-stimulated AC1 activity was examined at several concentrations of A23187 (Figure 4A) and extracellular Ca^{2+} (Figure 4B) to determine if somatostatin affected the Ca^{2+} sensitivity of AC1. Ca^{2+}-stimulated AC1 activity was inhibited by approximately 50% at all concentrations of A23187 and Ca^{2+} examined. These data indicate that somatostatin inhibits AC1 without affecting its Ca^{2+} sensitivity.

Pertussis Toxin Abolished Inhibition of AC1 by G_i-Coupled Receptors- To determine whether inhibition of AC1 by somatostatin or dopamine is mediated via the G_i/G_o class of G proteins, HEK 293 cells expressing AC1 were treated with pertussis toxin. This toxin catalyzes the ADP-ribosylation of the α subunit of G_i/G_o (Burns et al., 1983; Hsia et al., 1984; Katada and Ui, 1982; Katada and Ui, 1982; Neer et al., 1984) and uncouples G_i/G_o from its receptors (West et al., 1985). Somatostatin inhibition of AC1 was completely abolished by pertussis toxin (Figure 5A). Furthermore, dopaminergic inhibition of Ca^{2+}-stimulated AC1 was pertussis toxin sensitive (Figure 5B). Similar results were obtained when carbachol was used to stimulate AC1 (Figures 6A,B). These data indicate that somatostatin or dopamine inhibition of AC1 is most likely mediated by G_i or G_o.

Cellular expression of $\beta\gamma$-binding proteins did not affect G_i-coupled receptor inhibition of AC1- Because Ca^{2+} stimulation of AC1 was inhibited by G_i-coupled receptors *in vivo*, we were interested in assessing the role of the G-protein $\beta\gamma$ subunits in hormonally-driven inhibition since both $G_{i\alpha}$ and $\beta\gamma$ inhibit AC1 *in vitro* (Tang et al., 1991; Tang and G., 1991; Taussig et al., 1994; Tota et al., 1990). To accomplish this, we carried out transient transfections of HEK 293 cells in which the COOH-terminal, $\beta\gamma$-binding region of β-adrenergic receptor kinase 1 (βARK1-ct) or the α subunit of human rod transducin ($G_{t\alpha}$) was coexpressed with AC1. AC4 was used as a positive control for the expression of $\beta\gamma$-

binding proteins. Since $G_{s\alpha}$ stimulation of AC4 is potentiated by $\beta\gamma$ (Gao and Gilman, 1991), effective expression of $\beta\gamma$-binding proteins should block this potentiation. Cellular expression of "peptide minigenes" encoding $\beta\gamma$-binding pleckstrin homology (PH) domains of various proteins attenuates $\beta\gamma$ effects on phospholipase C, AC2, and the MAP kinase pathway (Inglese et al., 1994; Koch et al., 1994; Luttrell et al., 1995). Additionally, $G_{t\alpha}$ is an effective scavenger of free $\beta\gamma$ subunits in intact cells (Federman et al., 1992; Tsu and Wong, 1996).

In transient transfection experiments in which the G_s-coupled 5HT7 receptor was cotransfected, somatostatin potentiated serotonin stimulation of AC4, presumably through release of $\beta\gamma$ from G_i (Figure 7A). Coexpression of βARK1-ct with AC4 attenuated somatostatin-mediated potentiation of serotonin-stimulated AC4 almost entirely (Figure 7A). Under the same transfection conditions, there was no effect of βARK1-ct expression on somatostatin inhibition of Ca^{2+}-stimulated AC1 (Figure 7B). Similarly, coexpression of $G_{t\alpha}$ with AC4 attenuated somatostatin potentiation of serotonin-stimulated AC4 (Figure 8A), while somatostatin inhibition of A23187-stimulated AC1 was unaffected by coexpression of $G_{t\alpha}$ (Figure 8B). These results indicate that the primary mechansim for G_i-mediated inhibition of AC1 was through $G_{i\alpha}$ rather than $\beta\gamma$ release from dissociating G_i/G_o heterotrimers. However, we cannot rule out the possibility that a βARK1-ct-$\beta\gamma$ or $G_{t\alpha}$-$\beta\gamma$ complex was still capable of modulating AC1 but not AC4.

Coexpression of $\beta\gamma$-binding proteins elicited a modest stimulation of AC1 by G_s-coupled receptor activation- Activation of G_s-coupled receptors does not stimulate AC1 *in vivo* unless intracellular Ca^{2+} is simultaneously elevated (Wayman et al., 1994). Since $G_{s\alpha}$ stimulation of AC1 *in vitro* can be inhibited by $\beta\gamma$ (Tang et al., 1991; Tang and G., 1991; Taussig et al., 1994; Taussig et al., 1993; Tota et al., 1990) and since receptor activation of

G_S releases βγ, is it possible that the insensitivity of AC1 to G_S-coupled receptor activation is due to βγ inhibition? To address this issue, we examined the sensitivity of AC1 to activation of the G_S-coupled 5HT7 receptor *in vivo* was examined when βγ-binding proteins were coexpressed. Coexpression of $G_{t\alpha}$ with AC1 elicited a substantial (~4-fold) stimulation of AC1 by serotonin (Figure 9B). Experiments in which βARK1-ct was used as the βγ scavenger gave similar results (Figure 10B). In both cases, effective expression of βγ scavengers was determined with AC4 as described (see Figures 9A,10A). These data suggest that βγ release from dissociating G_S heterotrimers inhibits AC1.

Ca^{2+}-Stimulation of AC8 was not inhibited by G_i-Coupled Receptors- To determine whether Ca^{2+} stimulation of AC8 is also regulated by G_i-activating hormones, AC8 expressing cells were treated with 500nM somatostatin in the presence of increasing concentrations of A23187. AC8 was stimulated approximately 7-fold by 10μM A23187 in the presence of 1.8mM extracellular Ca^{2+} (Figure 11A). Concentrations of somatostatin as high as 500nM, which inhibited AC1, did not inhibit AC8 at any concentration of A23187 (Figure 11A) or extracellular Ca^{2+} (Figure 11B) examined. Stimulation of AC8 *in vivo* by forskolin was also insensitive to somatostatin (Figure 12).

To determine if AC8 is insensitive to other G_i-coupled receptors, cells stably coexpressing the D2L dopamine receptor with AC8 were treated with increasing concentrations of dopamine in the presence or absence of A23187. Ca^{2+}-stimulated AC8 was only inhibited 15% by 1μM dopamine (Figure 13). The insensitivity to somatostatin but slight inhibition by dopamine probably reflects differences in receptor density. The density of endogenous somatostatin receptors in HEK 293 cells is approximately 18 fmol/mg protein (Law, 1993), while the D2L cells used in this study express ~1500-2000 fmol/mg protein (V. Watts and K. Neve, personal communication). The D2L receptor density in the

cell lines used in this study was approximately ten-fold higher than the D2 receptor density in the striatum, which express the highest levels of D2 receptors in the brain (Yokoyama, 1994). Since the same stock of HEK 293 cells or dopamine D2L receptor expressing cells was used for expression of AC1 or AC8, variation in the number of somatostatin or D2L receptors present in AC1 or AC8 expressing cells cannot account for the difference in G_i sensitivity. Furthermore, somatostatin did not inhibit AC8 in four independent HEK 293 cell lines stably expressing AC8. These data strongly suggest that AC8 is insensitive to G_i-coupled receptor stimulation *in vivo*.

G_S and Ca^{2+} did not synergize to stimulate AC8- Since AC1 and AC8 respond quite differently to G_i-coupled receptors in HEK 293 cells, it was of interest to compare the sensitivites of these two enzymes to G_S-coupled receptors and Ca^{2+} *in vivo*. Both AC1 and AC8 were stimulated by Ca^{2+} alone but neither was activated by isoproterenol, a β-adrenergic agonist (Figure 14). AC1 was stimulated by isoproterenol when intracellular Ca^{2+} was elevated with A23187 (Figure 14A). In contrast, isoproterenol did not produce any additional stimulation of AC8 beyond that caused by A23187 alone (Figure 14B). Stimulation of AC8 by coapplication of Ca^{2+} and isoproterenol was strongly inhibited by somatostatin whereas it had no effect on AC8 activities (data not shown).

Somatostatin inhibited Ca^{2+}-stimulated cAMP accumulation in primary cortical and hippocampal neuron cultures-Since AC1 is neural specific (Xia et al., 1993) and expressed in the cortex and hippocampus (Xia et al., 1991), we were interested in determining whether or not somatostatin inhibits Ca^{2+}-stimulated cAMP levels in cultured neurons. Primary cultures of cortical and hippocampal neurons were prepared from day one rat pups. Treatment of these cultures with 1μM somatostatin produced a substantial inhibition of

cAMP levels stimulated with 1μM A23187 demonstrating that Ca^{2+}-stimulated cAMP synthesis in the hippocampus and cortex is sensitive to G_i-coupled hormones (Figure 15) .

Discussion

Although Ca^{2+} stimulation of specific adenylyl cyclases in brain may contribute to neuroplasticity (Abrams et al., 1991; Levin et al., 1992; Livingston et al., 1984; Weisskopf et al., 1994; Wu et al., 1995; Xia et al., 1991), mechanisms for inhibition of adenylyl cyclases may be equally important. In most cells, increases in cAMP in response to extracellular and intracellular signals are transient. Mechanisms for attenuation of cAMP signals include inhibition of adenylyl cyclases by G_i-coupled receptors (Taussig and Gilman, 1995), CaM kinase inhibition of specific adenylyl cyclases (Wayman et al., 1995) and cAMP hydrolysis by phosphodiesterases (Beavo, 1995). The objective of this study were to determine if AC1 or AC8 is inhibited by G_i-coupled receptors *in vivo* .

In this study, we discovered that AC1 is inhibited *in vivo* by activation of G_i-coupled receptors including somatostatin and D2L dopamine receptors. Inhibition was apparently mediated through the G_i/G_o class of heterotrimeric G proteins since it was blocked by pertussis toxin. In particular, we presume that inhibition was mediated by G_i because $G_{i\alpha 1}$ and $G_{i\alpha 3}$ are expressed at relatively high levels in HEK 293 cells compared to $G_{o\alpha 2}$ (Law, 1993). Furthermore, $G_{o\alpha}$ inhibition of AC1 *in vitro* is 10-fold less potent than that elicited by $G_{i\alpha}$ (Taussig et al., 1994). Mechanisms for receptor-coupled inhibition of AC1 *in vivo* have not been defined but could include contributions from activated $G_{i\alpha}$ and/or $\beta\gamma$ since both have been shown to inhibit AC1 *in vitro* (Tang et al., 1991; Taussig et al., 1993; Taussig et al., 1994; Taussig et al., 1993; Tota et al., 1990). Our data suggest that receptor stimulated inhibition of AC1 is due primarily to activated $G_{i\alpha}$ since

coexpression of βγ-binding proteins did not prevent somatostatin inhibition. Parallel experiments with AC4, coactivated with serotonin and somatostatin illustrated that βγ was effectively inhibited by coexpression of the same βγ-binding proteins, βARK1-ct or $G_{t\alpha}$.

Release of inhibitory βγ from G_s may explain why AC1 is not activated by G_s-coupled receptors *in vivo*. In fact, sequestration of βγ released from G_s caused stimulation of AC1 by G_s-coupled receptor activation *in vivo*. The differing effects of βγ sequestration following G_s versus G_i receptor activation could be because of distinct βγ subunit compositions for G_s or G_i. Interestingly, using the yeast two-hybrid system, Yan and Gautam have shown that there are substantial differences in the interactions between AC2 and β1-5 (Yan, 1996). In particular, β1 interacts with AC2 3-4 times more strongly than β4, and at least twice as strong as β3 and β5. It may be that different β isoforms determine, in large part, the potency with which a given βγ complex affects adenylyl cyclases *in vivo*. Alternatively, βγ release from G_i may be functionally inconsequential since the the dominant mechanism for G_i-mediated inhibition is through the release $G_{i\alpha}$. In any case, these data indicate that βγ and $G_{i\alpha}$ can both inhibit AC1 activity in intact cells.

Mammalian brain expresses at least two CaM-stimulated adenylyl cyclases, AC1 and AC8, both of which are activated by intracellular Ca^{2+}. These enzymes are expressed in the hippocampus (Cali et al., 1994; Xia et al., 1991), and AC1 knockout mice show residual Ca^{2+}-stimulated adenylyl cyclase activity consistent with the properties of AC8 (Villacres et al., 1995; Wu et al., 1995). Surprisingly, AC8 was not inhibited by activation of somatostatin receptors or high levels of exogenously expressed dopamine D2L receptors. In addition, AC8 was not stimulated by G_s-coupled receptors *in vivo* in the presence or absence of elevated intracellular Ca^{2+}. A comparison of the regulatory properties of AC1 and AC8 indicates that they differ in Ca^{2+} sensitivity, stimulation by G_s-coupled receptors, and inhibition by G_i-coupled receptors or CaM-dependent protein kinase IV (Table III). The

differences in Ca^{2+} sensitivity are particularly interesting since AC1 is stimulated by very low intracellular Ca^{2+} (150 to 200nM) while AC8 is much less sensitive to Ca^{2+} (700 to 800nM). AC8 is apparently regulated only by intracellular Ca^{2+}*in vivo*.

The regulatory properties of AC1 suggest that it may produce transient cAMP increases in response to relatively low intracellular Ca^{2+} signals. AC8 may generate more prolonged cAMP signals in response to robust Ca^{2+} increases since its activity is not inhibited by G_i-coupled receptors or CaM-kinases. These distinct signaling mechanisms may be important for different forms of synaptic plasticity. For example, it has been proposed that AC1 may play a role in mf-CA3 LTP by coupling presynaptic Ca^{2+} elevations to cAMP increases which enhances glutamate release (Weisskopf et al., 1994). In fact, mutant mice lacking AC1 show greatly depressed mf-CA3 LTP (Villacres and Storm, unpublished observations) but normal long-lasting LTP (L-LTP) in the CA1 region of hippocampus. L-LTP is dependent upon cAMP-dependent protein kinase (Frey et al., 1993; Matthies et al., 1990) and transcription (Nguyen et al., 1994). The stimulus paradigm that generates L-LTP stimulates cAMP response element (CRE)-mediated transcription in area CA1 of the hippocampus (Impey, 1996). Since cAMP stimulation of transcription may depend upon prolonged cAMP increases (Backsai et al., 1993; Hagiwara et al., 1993), AC8 may play an important role in L-LTP that depends upon cAMP-stimulated transcription.

In summary, the two Ca^{2+}-stimulated brain adenylyl cyclases, AC1 and AC8 are not redundant enzyme activities. They show very distinct regulatory properties that may be important for specific forms of synaptic plasticity in the brain. The physiological function of these enzymes and the relationship between their regulatory properties and neuroplasticity may become apparent when mutant mice lacking AC8 become available.

Figure 3. G_i-coupled receptors inhibited Ca^{2+}/calmodulin-stimulated AC1 in HEK 293 cells. (A) HEK 293 cells expressing AC1 (indicated as AC1 in subsequent figures) were treated with the indicated concentrations of somatostatin and either EtOH vehicle or 5μM A23187. (B) HEK 293 cells expressing the dopamine D2L receptor with AC1 (indicated as D2L/AC1 in subsequent figures) were treated with the indicated concentrations of dopamine in the presence of 5μM A23187 or vehicle. HEK 293 cells do not contain endogenous Ca^{2+}-stimulated adenylyl cyclase activity and are not represented. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/- SD of triplicate assays.

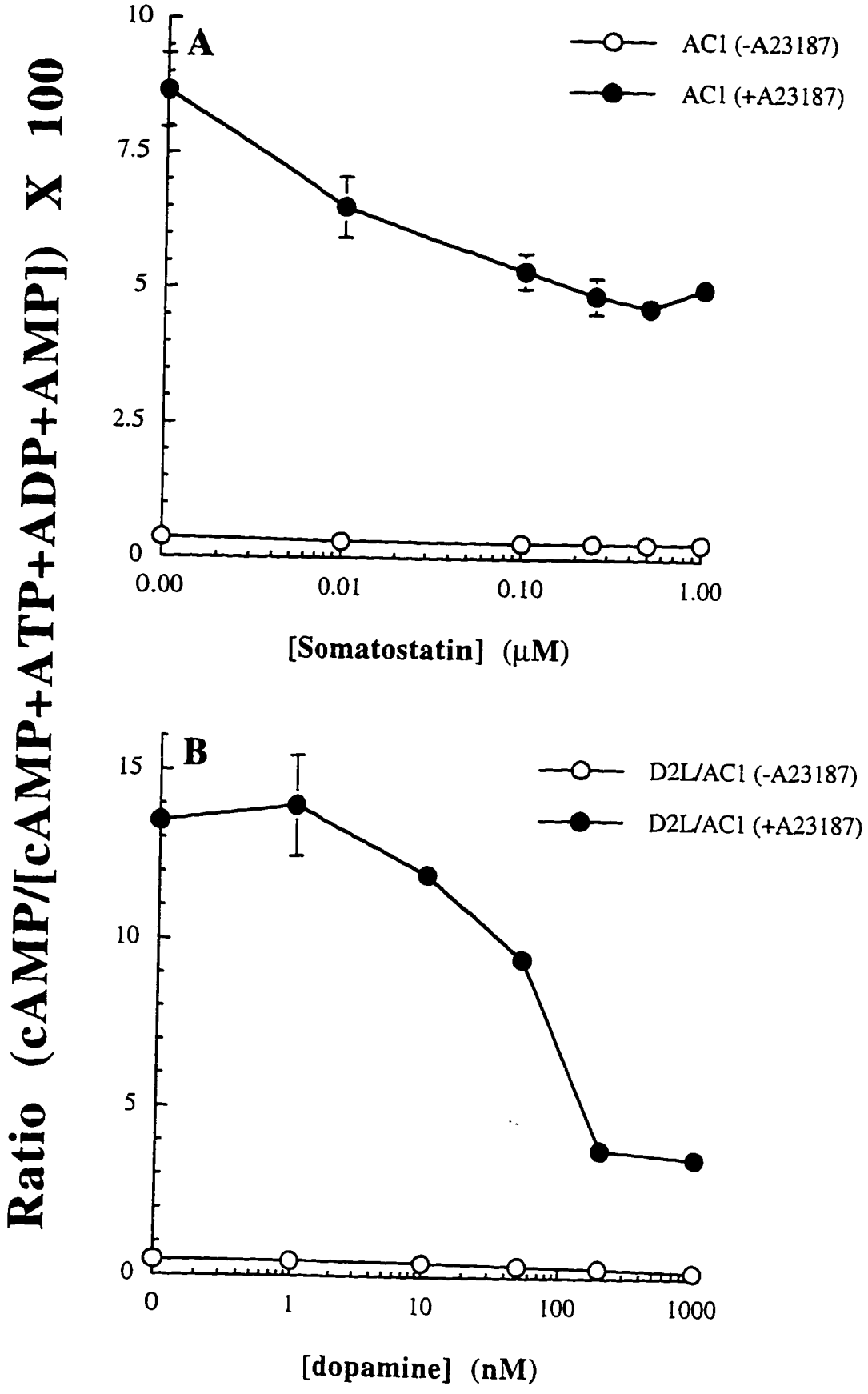

A
AC1 (-A23187)
AC1 (+A23187)
10
7.5
5
2.5
0
0.00
0.01
0.10
1.00
[Somatostatin] (μM)
B
D2L/AC1 (-A23187)
D2L/AC1 (+A23187)
15
10
5
0
0
1
10
100
1000
[dopamine] (nM)
Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100

Figure 4. Somatostatin inhibited AC1 in HEK 293 cells without altering the EC_{50} for Ca^{2+}. (A) HEK 293 cells expressing AC1 were treated with the indicated concentrations of A23187 in the presence or absence of 1μM somatostatin. Extracellular Ca^{2+} was 2mM. (B) HEK 293 cells expressing AC1 were assayed in the presence of 7.5μM A23187, varying concentrations of extracellular Ca^{2+}, with or without 500nM somatostatin. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/- SD of triplicate assays.

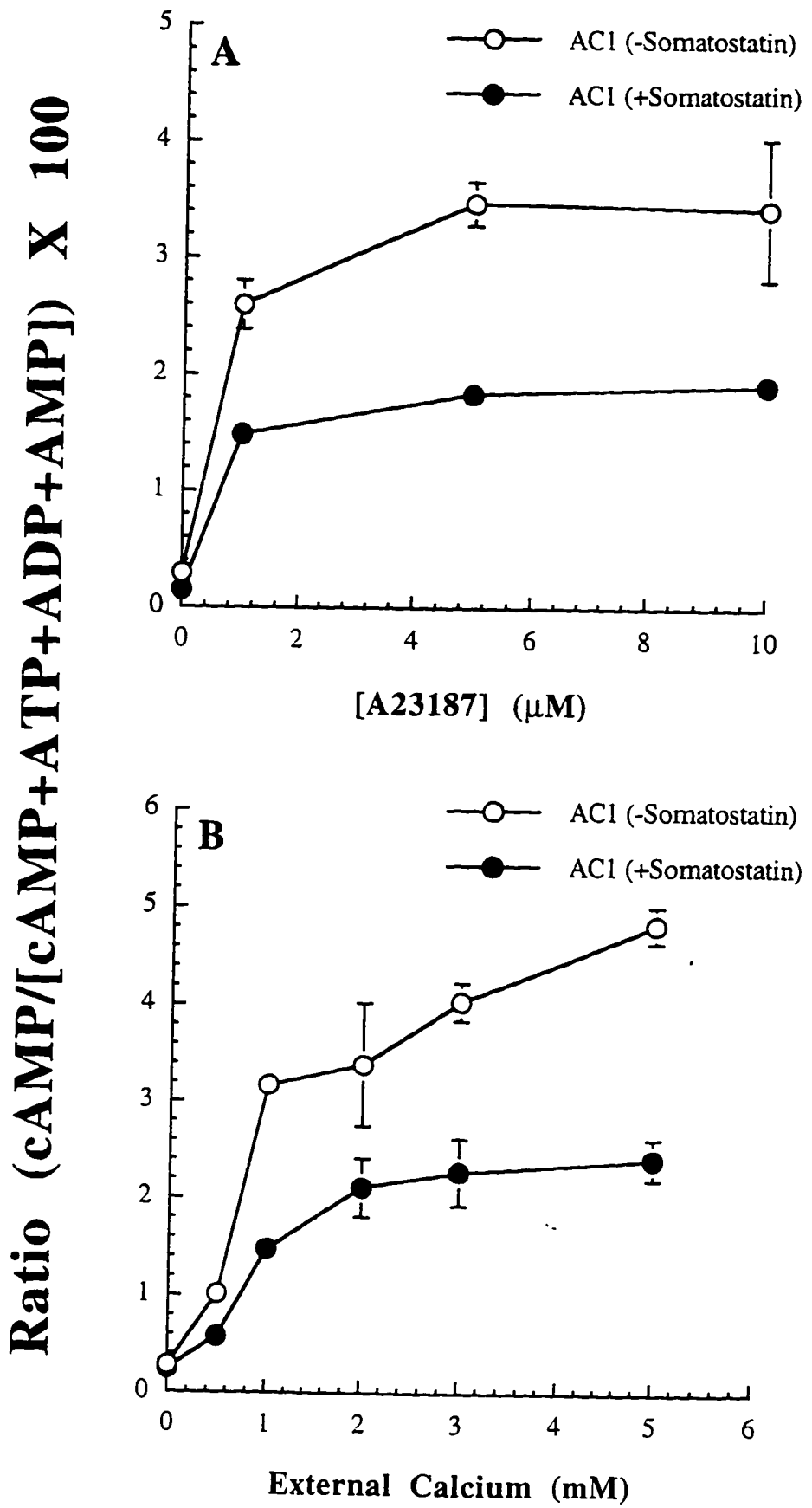

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
AC1 (-Somatostatin)
AC1 (+Somatostatin)
0
1
2
3
4
5
0
2
4
6
8
10
[A23187] (μM)
B
AC1 (-Somatostatin)
AC1 (+Somatostatin)
0
1
2
3
4
5
6
0
1
2
3
4
5
6
External Calcium (mM)

Figure 5. Pertussis toxin abolished inhibition of A23187-stimulated AC1 by G_i-coupled receptors in HEK 293 cells. (A) HEK 293 cells expressing AC1 were incubated overnight with vehicle or 200ng/ml pertussis toxin (PTx). The following day, cells were treated with the indicated concentrations of A23187 in the presence or absence of 500nM somatostatin (SOM). Extracellular Ca^{2+} was 2mM. (B) HEK 293 cells coexpressing D2L and AC1 were incubated overnight with vehicle or 200ng/ml pertussis toxin. The following day, cells were treated with 5μM A23187 in the presence or absence of 1μM dopamine. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/- SD of triplicate assays.

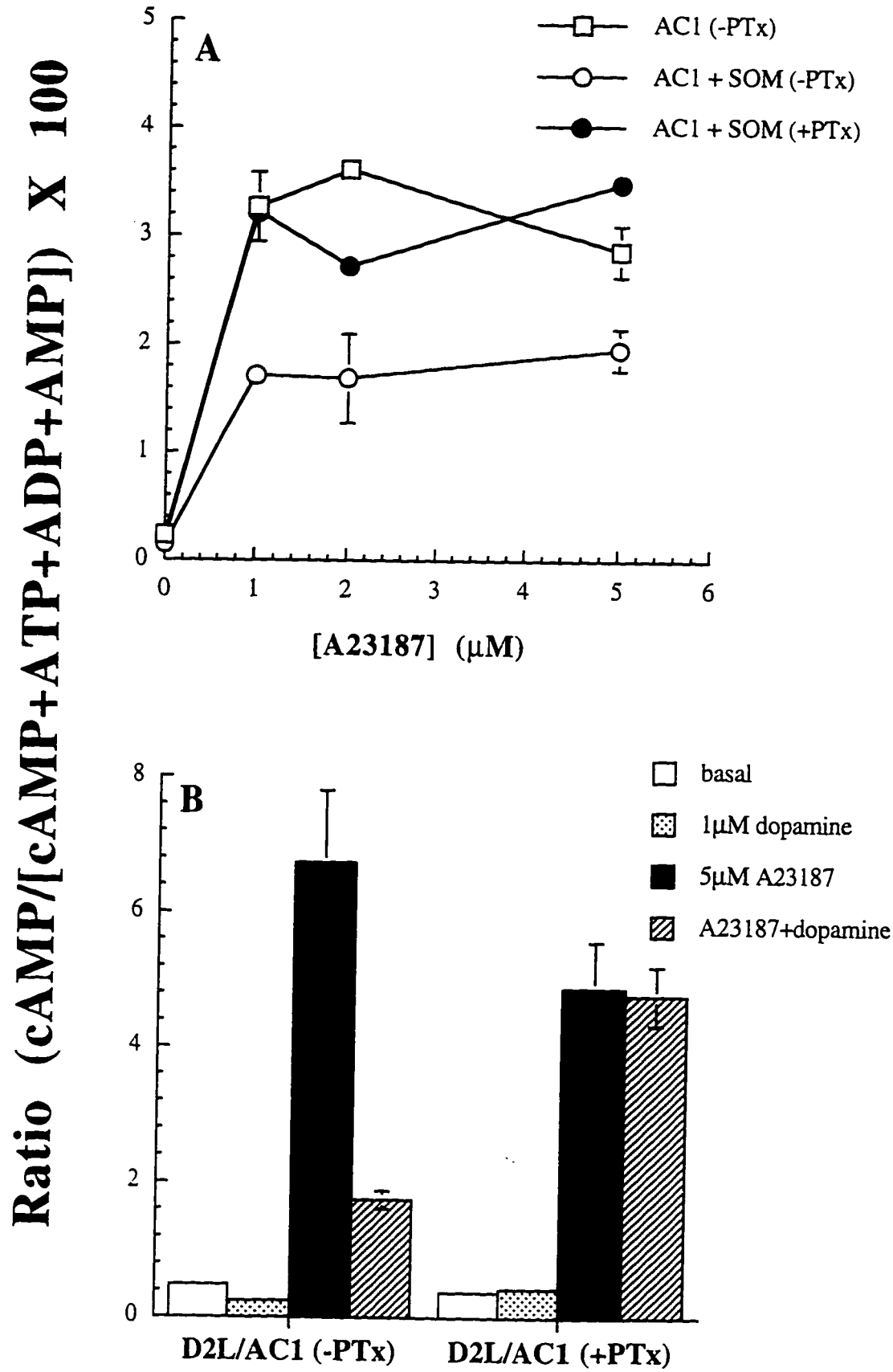

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
AC1 (-PTx)
AC1 + SOM (-PTx)
AC1 + SOM (+PTx)
0
1
2
3
4
5
6
[A23187] (μM)
B
basal
1μM dopamine
5μM A23187
A23187+dopamine
0
2
4
6
8
D2L/AC1 (-PTx)
D2L/AC1 (+PTx)

Figure 6. Pertussis toxin abolished somatostatin inhibition of AC1 stimulated by muscarinic receptor-mediated increases in intracellular Ca^{2+}. HEK 293 cells expressing AC1 were incubated overnight with vehicle (A) or 200ng/ml pertussis toxin (B). The following day, cells were treated with the indicated concentrations of carbachol in the presence or absence of 500nM somatostatin. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/- SD of triplicate determinations.

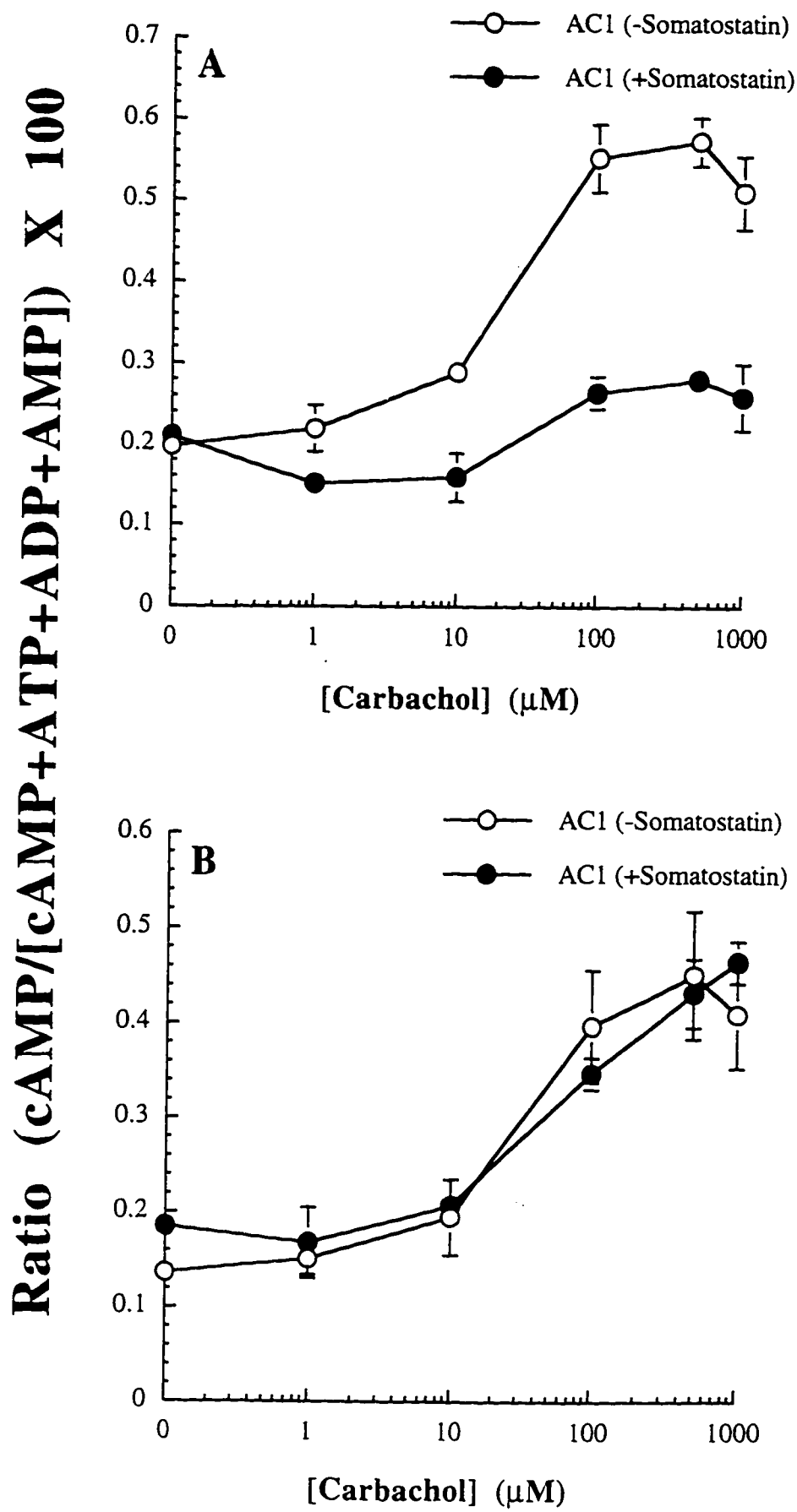
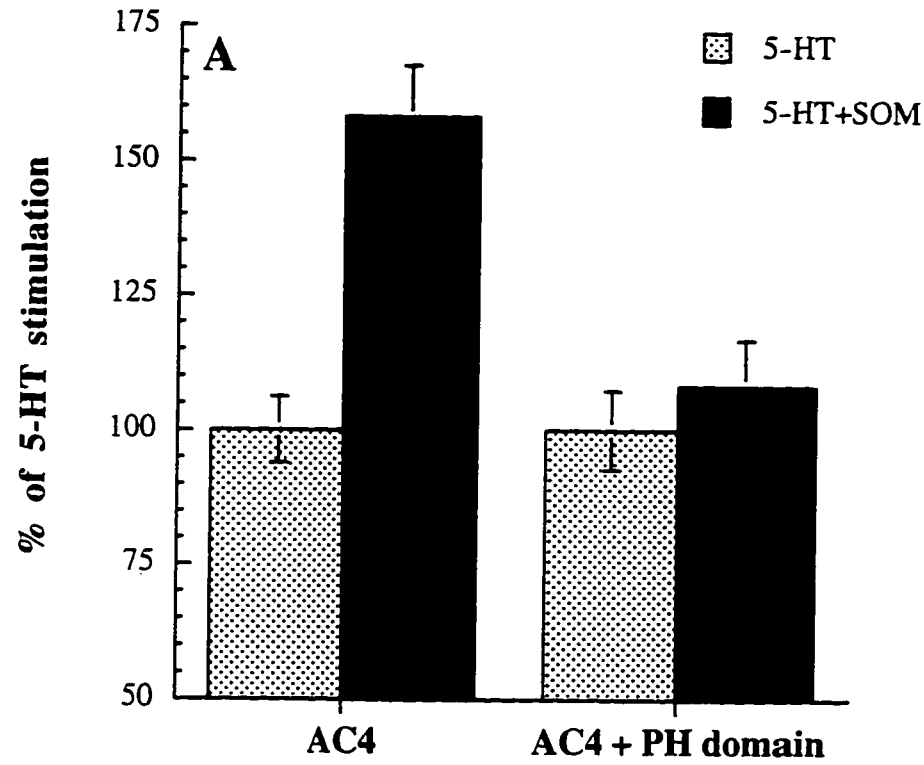

A
AC1 (-Somatostatin)
AC1 (+Somatostatin)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
10
100
1000
[Carbachol] (μM)
B
AC1 (-Somatostatin)
AC1 (+Somatostatin)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
10
100
1000
[Carbachol] (μM)
Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100

Figure 7. Effect of βARK1-ct minigene expression on somatostatin inhibition of AC1. HEK 293 cells were transiently transfected with both RSV-β-galactosidase and the 5HT7 receptor, and either pCDNAIII or βARK1-ct, as well as either pCEP4, AC1, or AC4 as described in Materials and Methods. Cells transfected with βARK1-ct are denoted as "PH domain". (Panel A) AC4-transfected cells with or without βARK1-ct coexpressed were treated with 10μM serotonin (5HT) in the presence or absence of 500nM somatostatin (SOM). (Panel B) AC1-transfected cells with or without βARK1-ct coexpressed were treated with 5μM A23187 in the presence or absence of 500nM somatostatin (SOM). Relative cAMP accumulation was determined as described in Materials and Methods. Data are expressed as % cAMP accumulation in the absence of somatostatin with this level being set as 100%. The fold stimulation over the basal activities was similar between transfections with or without βARK1-ct. The data are the mean +/-SD of triplicate assays and are subtracted for endogenous (pCEP4 transfectants) cAMP accumulation and corrected for transfection efficiency using β-galactosidase expression.


A
175
150
125
100
75
50
% of 5-HT stimulation
5-HT
5-HT+SOM
AC4
AC4 + PH domain

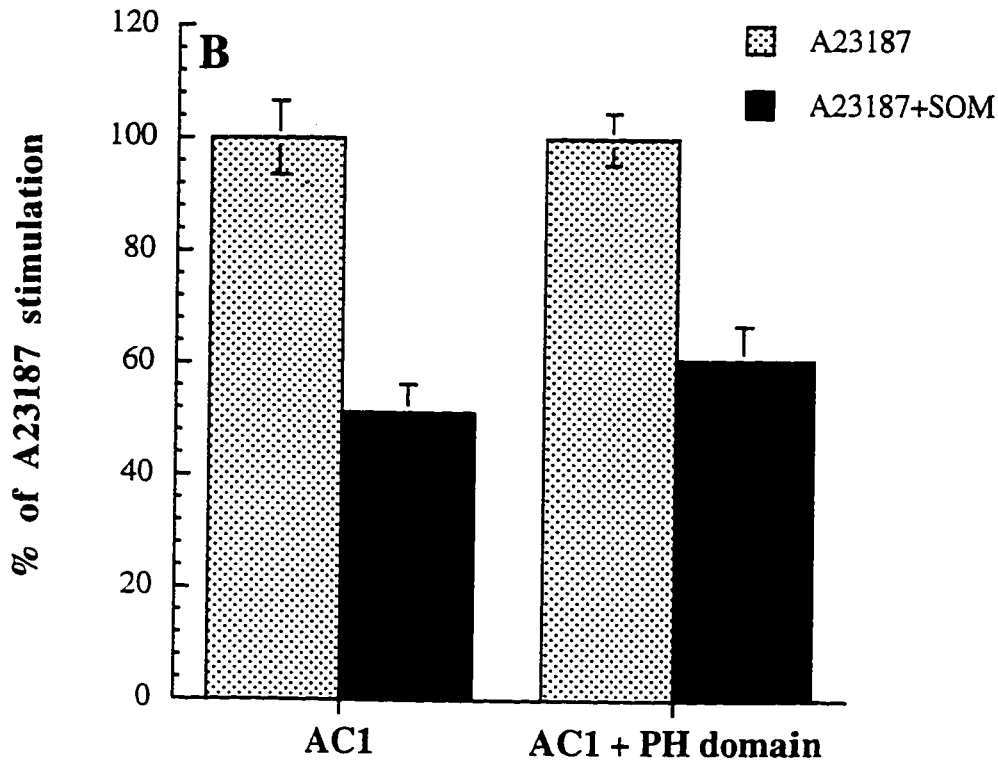
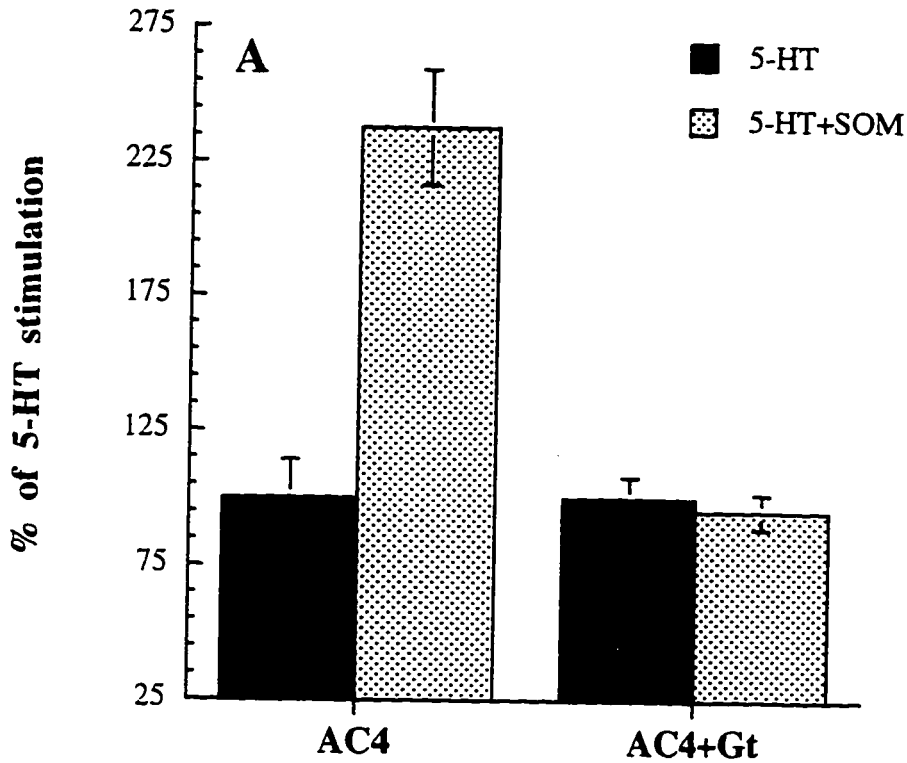

B
120
100
80
60
40
20
0
% of A23187 stimulation
A23187
A23187+SOM
AC1
AC1 + PH domain

Figure 8. Effect of transducin-α expression on somatostatin inhibition of AC1. HEK 293 cells were transiently transfected with both RSV-β-galactosidase and the 5HT7 receptor, and either pCDNAIII or $G_{t\alpha}$, as well as either pCEP4, AC1, or AC4 as described in Materials and Methods. Cells transfected with $G_{t\alpha}$ are denoted as "G_t". (Panel A) AC4-transfected cells with or without $G_{t\alpha}$ coexpressed were treated with 10μM serotonin (5HT) in the presence or absence of 500nM somatostatin (SOM). (Panel B) AC1-transfected cells with or without $G_{t\alpha}$ coexpressed were treated with 5μM A23187 in the presence or absence of 500nM somatostatin (SOM). Data are expressed as % cAMP accumulation in the absence of somatostatin with this level being set as 100%. The fold stimulation over the basal activities was similar between transfections with or without transducin-α. The data are the mean +/- SD of triplicate determinations and are subtracted for endogenous (pCEP4 transfectants) cAMP accumulation and corrected for transfection efficiency using β-galactosidase expression.


A
5-HT
5-HT+SOM
% of 5-HT stimulation
275
225
175
125
75
25
AC4
AC4+Gt

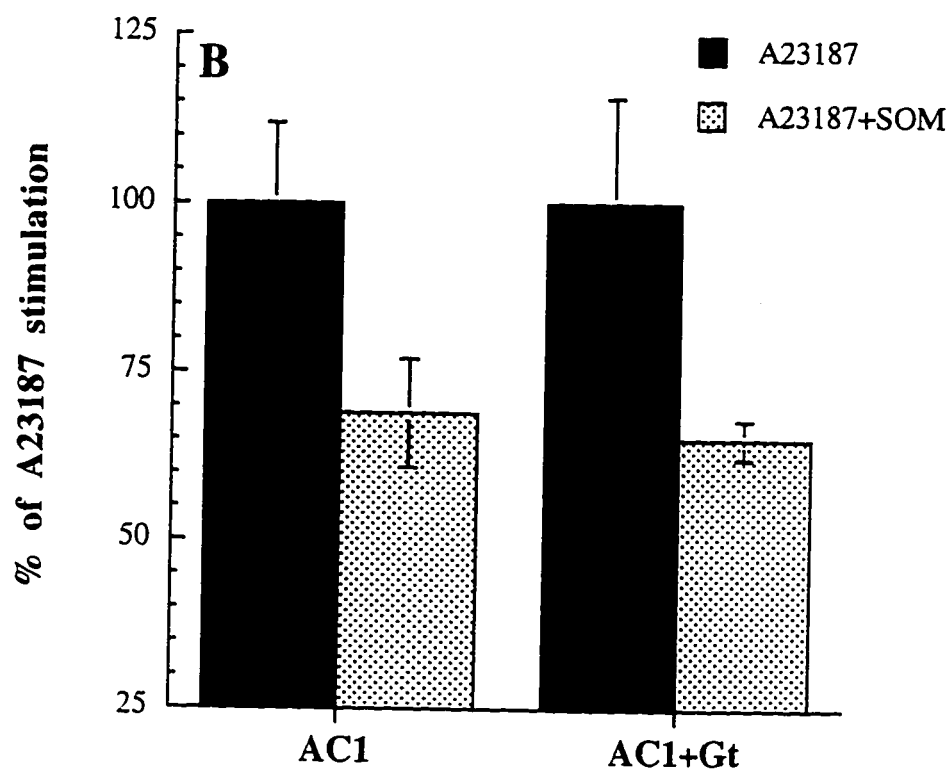
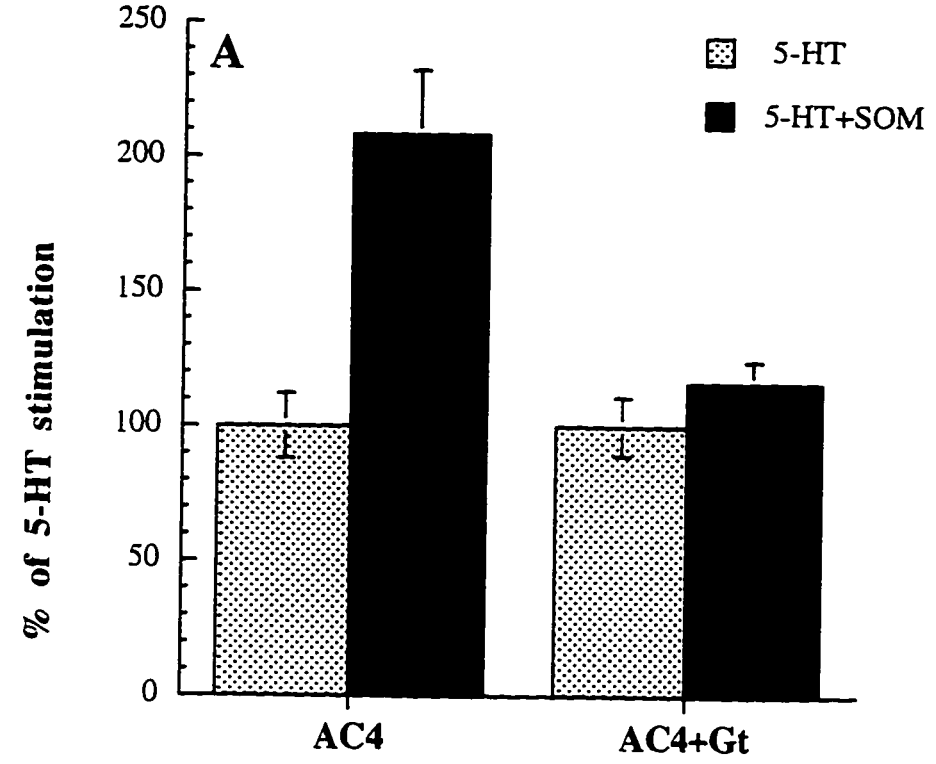

B
A23187
A23187+SOM
% of A23187 stimulation
125
100
75
50
25
AC1
AC1+Gt

Figure 9. Expression of transducin-α with AC1 generated stimulation by activation of the G_S-coupled 5HT7 receptor in vivo. HEK 293 cells were transiently transfected with both RSV-β-galactosidase and the 5HT7 receptor, and either pCDNAIII or $G_{t\alpha}$, as well as either pCEP4, AC1, or AC4 as described in Materials and Methods. Cells transfected with $G_{t\alpha}$ are denoted as "G_t". (Panel A) AC4-transfected cells with or without $G_{t\alpha}$ coexpressed were treated with 10μM serotonin (5HT) in the presence or absence of 500nM somatostatin (SOM). (Panel B) AC1-transfected cells with or without $G_{t\alpha}$ coexpressed were treated with or without 10μM serotonin (5HT). Data for AC4 are expressed as the % cAMP accumulation in the absence of somatostatin with this level being set as 100%. Data for AC1 are shown as % of basal cAMP accumulation with the baseline cAMP level set as 100%. The data are the mean +/- SD of triplicate determinations and are subtracted for endogenous cAMP levels and corrected for transfection efficiency using β-galactosidase.


A
5-HT
5-HT+SOM
% of 5-HT stimulation
250
200
150
100
50
0
AC4
AC4+Gt

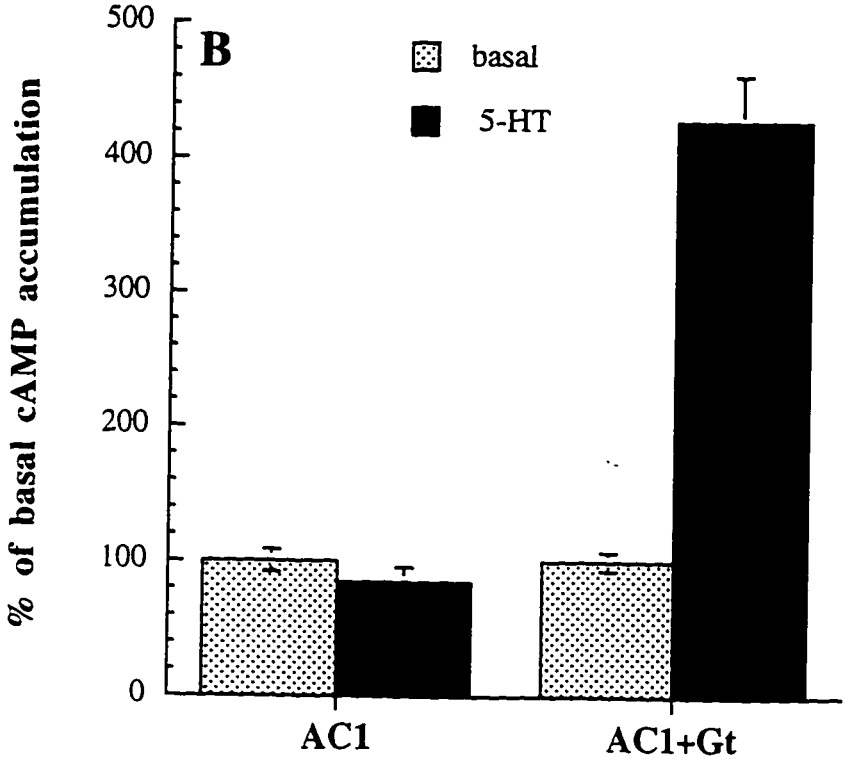
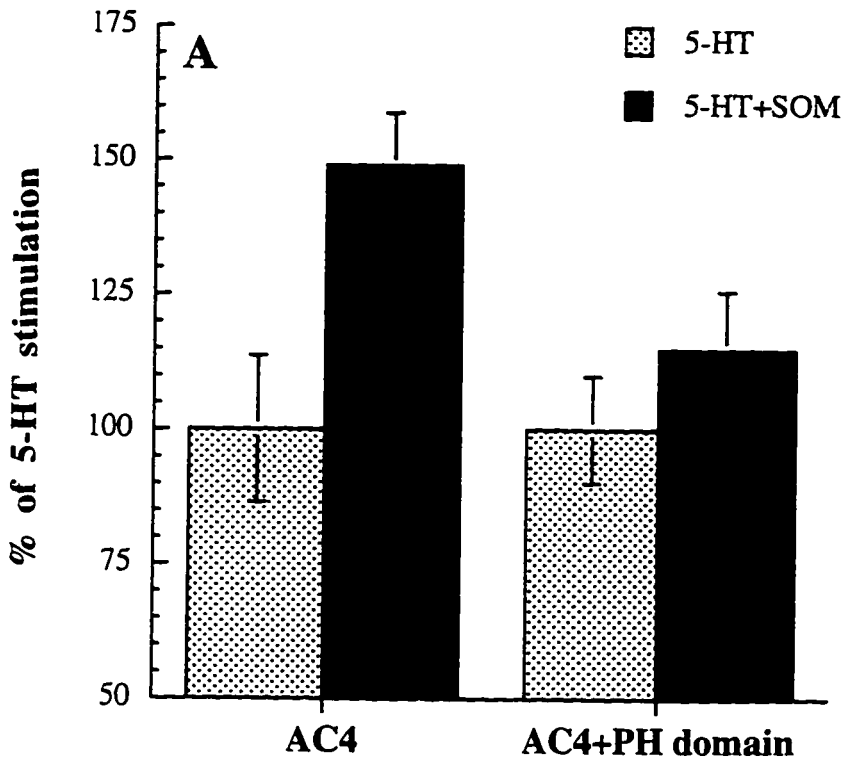

B
basal
5-HT
% of basal cAMP accumulation
500
400
300
200
100
0
AC1
AC1+Gt

Figure 10. Expression of βARK_1-ct with AC1 generated stimulation by activation of the G_S-coupled 5HT7 receptor in vivo. HEK 293 cells were transiently transfected with both RSV-β-galactosidase and the 5HT7 receptor, and either pCDNAIII or βARK_1-ct, as well as either pCEP4, AC1, or AC4 as described in Materials and Methods. Cells transfected with βARK_1-ct are denoted as "PH domain". (Panel A) AC4-transfected cells with or without βARK_1-ct coexpressed were treated with 10μM serotonin (5HT) in the presence or absence of 500nM somatostatin (SOM). (Panel B) AC1-transfected cells were treated with or without 10μM serotonin (5HT). Data for AC4 are expressed as the % cAMP accumulation in the absence of somatostatin with this level being set as 100%. Data for AC1 are shown as % of basal cAMP accumulation with the baseline cAMP level set as 100%. The data are the mean +/- SD of triplicate determinations and are subtracted for endogenous cAMP levels and corrected for transfection efficiency using β-galactosidase.


A
5-HT
5-HT+SOM
% of 5-HT stimulation
175
150
125
100
75
50
AC4
AC4+PH domain

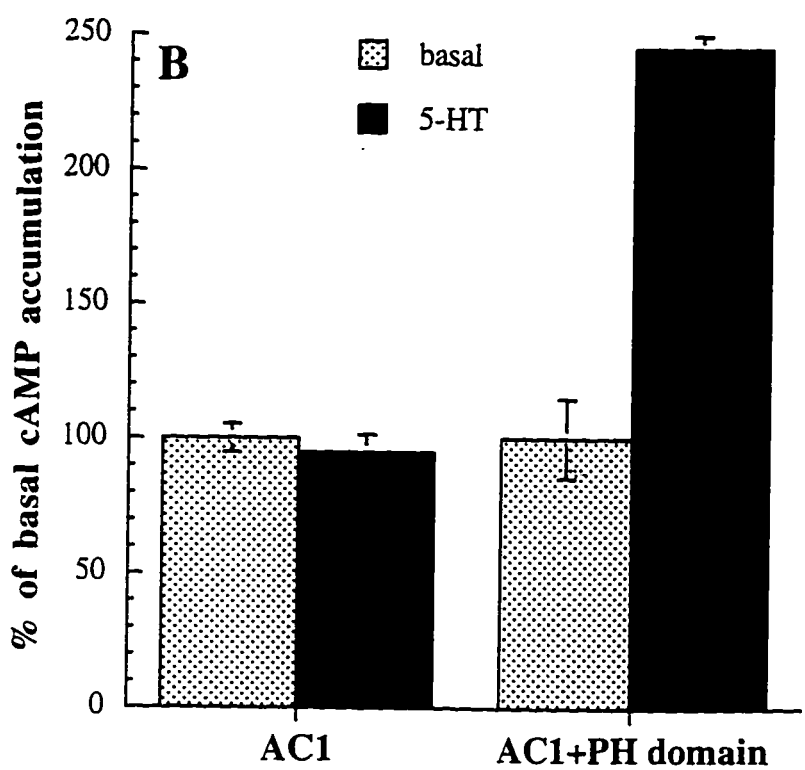

B
basal
5-HT
% of basal cAMP accumulation
250
200
150
100
50
0
AC1
AC1+PH domain

Figure 11. Somatostatin did not inhibit Ca^{2+}/calmodulin stimulation of AC8 in vivo. (A) HEK 293 cells expressing AC8 (designated as AC8) were treated with the indicated concentrations of A23187 in the presence or absence of 500nM somatostatin. Extracellular Ca^{2+} was 2mM. (B) HEK 293 cells expressing AC8 were treated with 7.5μM A23187, in the presence of varying concentrations of external Ca^{2+}, with or without 500nM somatostatin. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/- SD of triplicate assays.

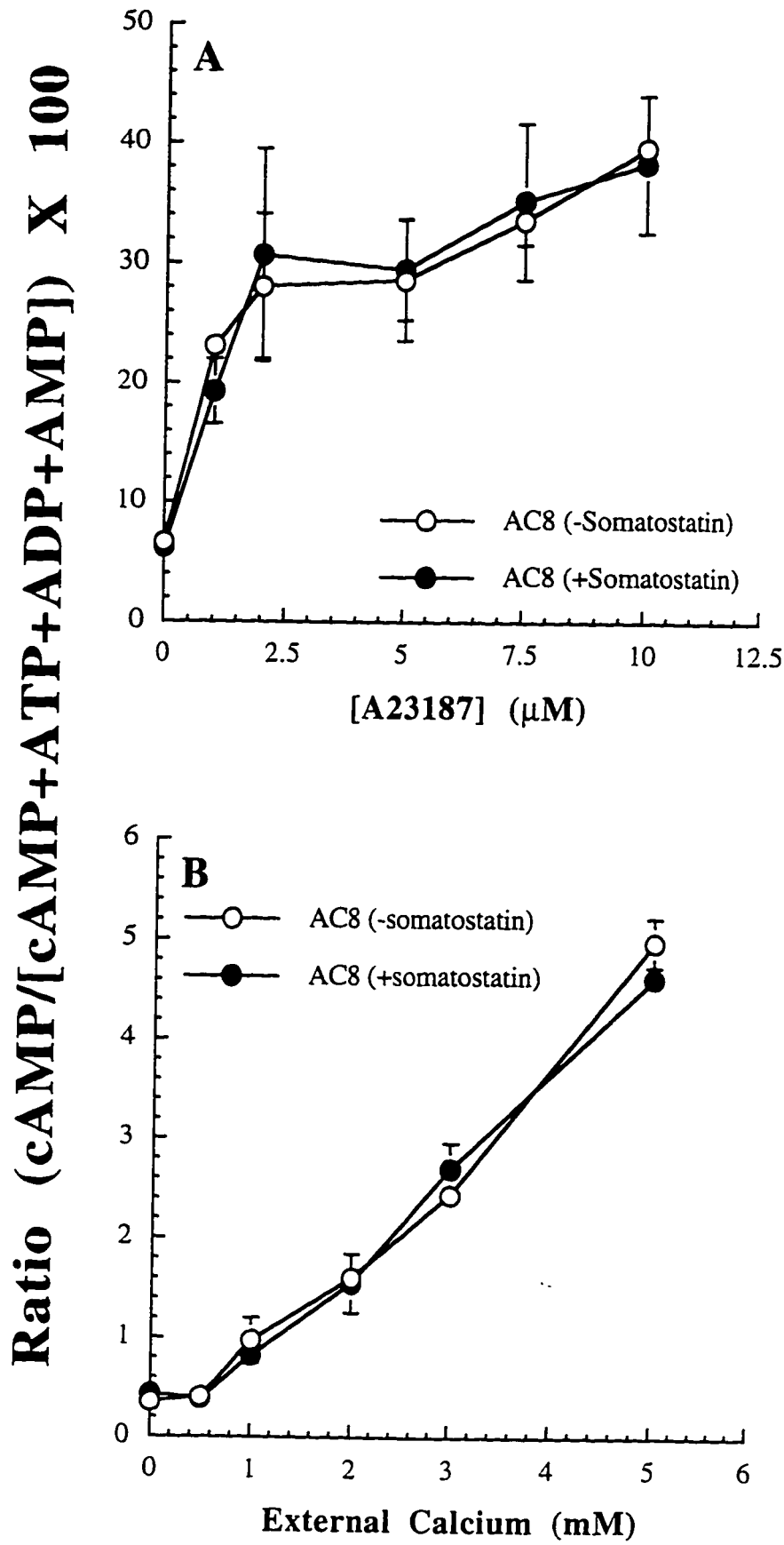

Ratio ([cAMP]/[cAMP+ATP+ADP+AMP]) X 100
A
50
40
30
20
10
0
0
2.5
5
7.5
10
12.5
AC8 (-Somatostatin)
AC8 (+Somatostatin)
[A23187] (μM)
B
6
5
4
3
2
1
0
0
1
2
3
4
5
6
AC8 (-somatostatin)
AC8 (+somatostatin)
External Calcium (mM)

Figure 12. Somatostatin does not inhibit forskolin-stimulation of AC8 in intact HEK 293 cells. (A) HEK 293 cells were treated with the indicated concentrations of somatostatin in the presence or absence of 1μM forskolin. (B) HEK 293 cells expressing AC1 were treated with the indicated concentrations of somatostatin in the presence or absence of 1μM forskolin. (C) HEK 293 cells expressing AC8 were treated with the indicated concentrations of somatostatin in the presence or absence of 1μM forskolin. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/-SD of triplicate assays.

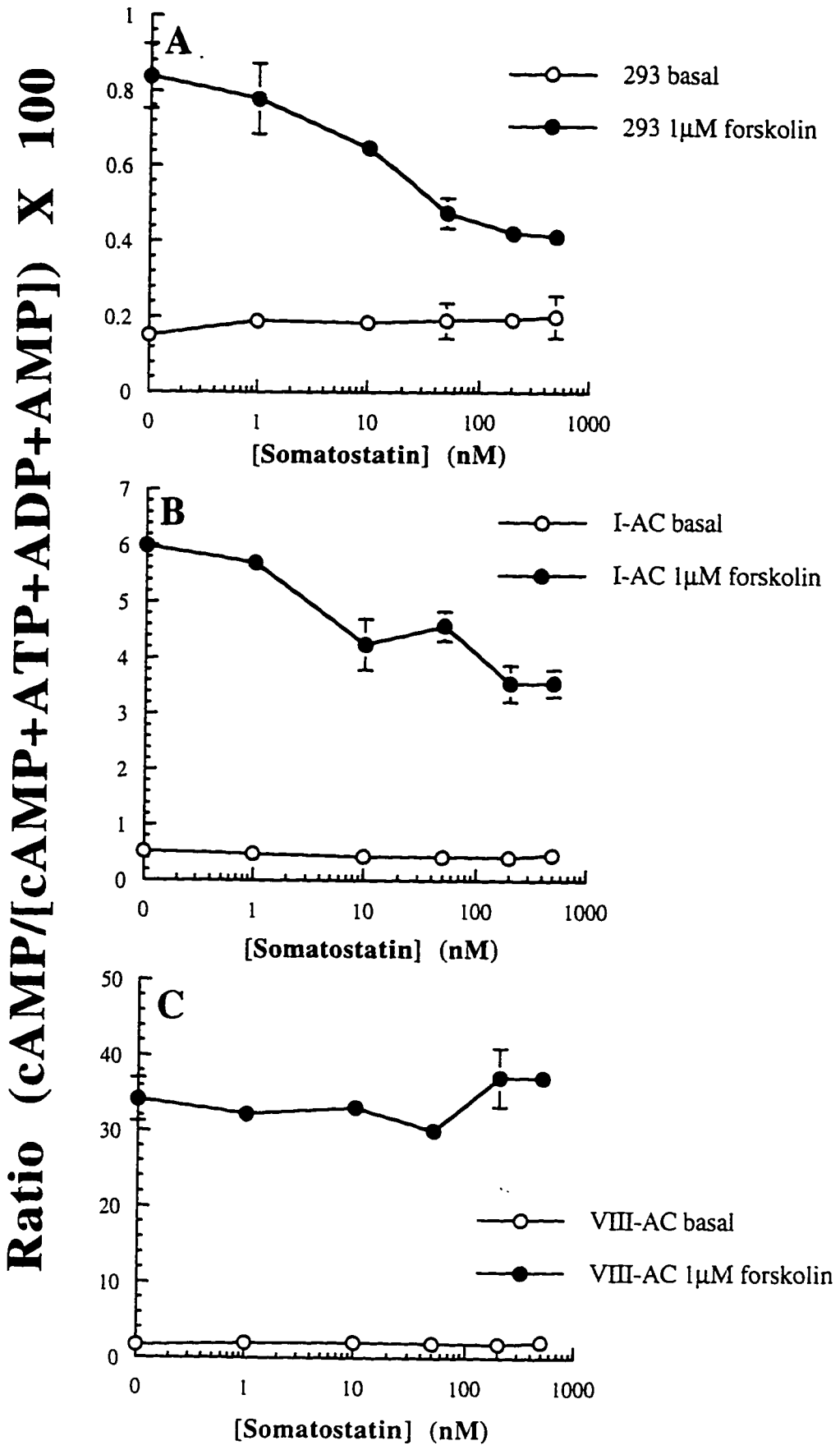

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
293 basal
293 1μM forskolin
[Somatostatin] (nM)
B
I-AC basal
I-AC 1μM forskolin
[Somatostatin] (nM)
C
VIII-AC basal
VIII-AC 1μM forskolin
[Somatostatin] (nM)

Figure 13. Dopamine did not inhibit Ca^{2+}/calmodulin stimulation of AC8 expressed in HEK 293 cells. HEK 293 cells coexpressing the D2L receptor and AC8 were treated with 5μM A23187 in the presence of the indicated concentrations of dopamine. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/- SD of triplicate determinations.

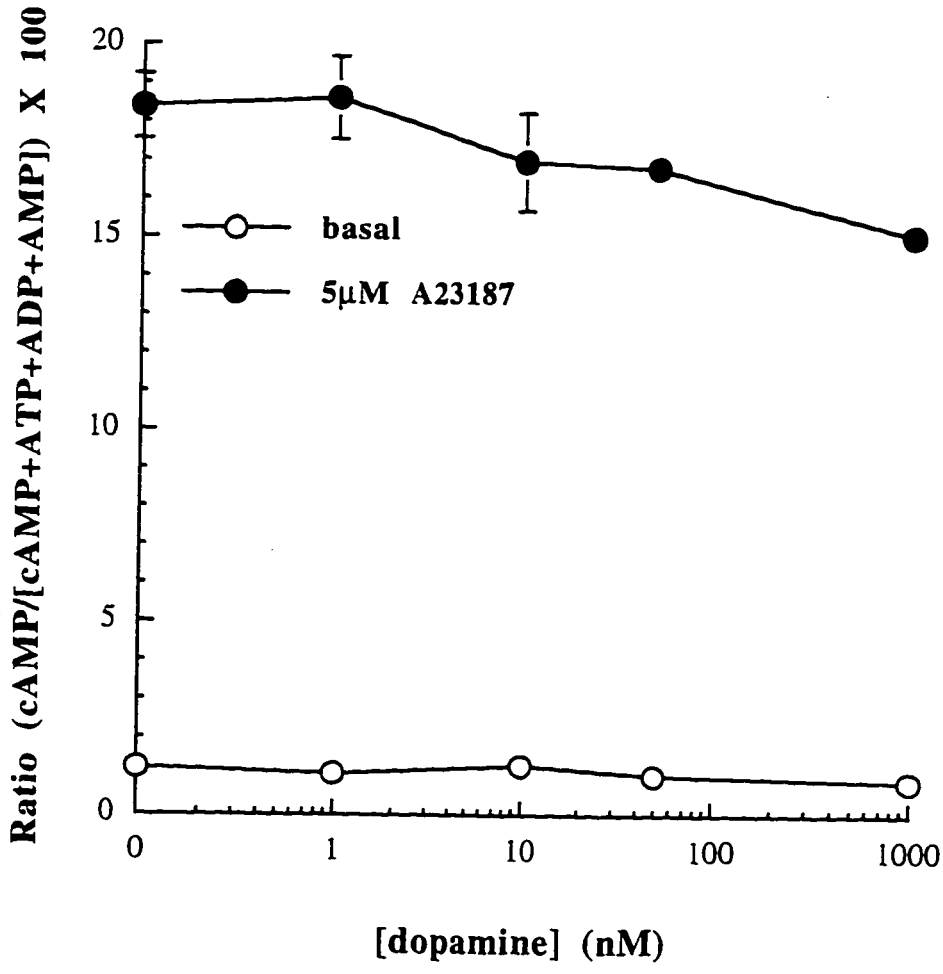

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
20
15
10
5
0
basal
5μM A23187
0
1
10
100
1000
[dopamine] (nM)

Figure 14. AC8 expressed in HEK 293 cells was not synergistically stimulated by Ca^{2+} and G_s-coupled receptor activation. HEK 293 cells expressing pCEP4 vector (designated 293 in the top and bottom panels), AC1 (top), or AC8 (bottom) were treated with vehicle, 10μM isoproterenol, 10μM A23187, or isoproterenol and A23187. Note that the HEK 293 cell cAMP levels presented are duplicated in A and B since they are from the same experiment. Relative cAMP accumulation was determined as described in Materials and Methods. The data are mean +/- SD or triplicate assays.

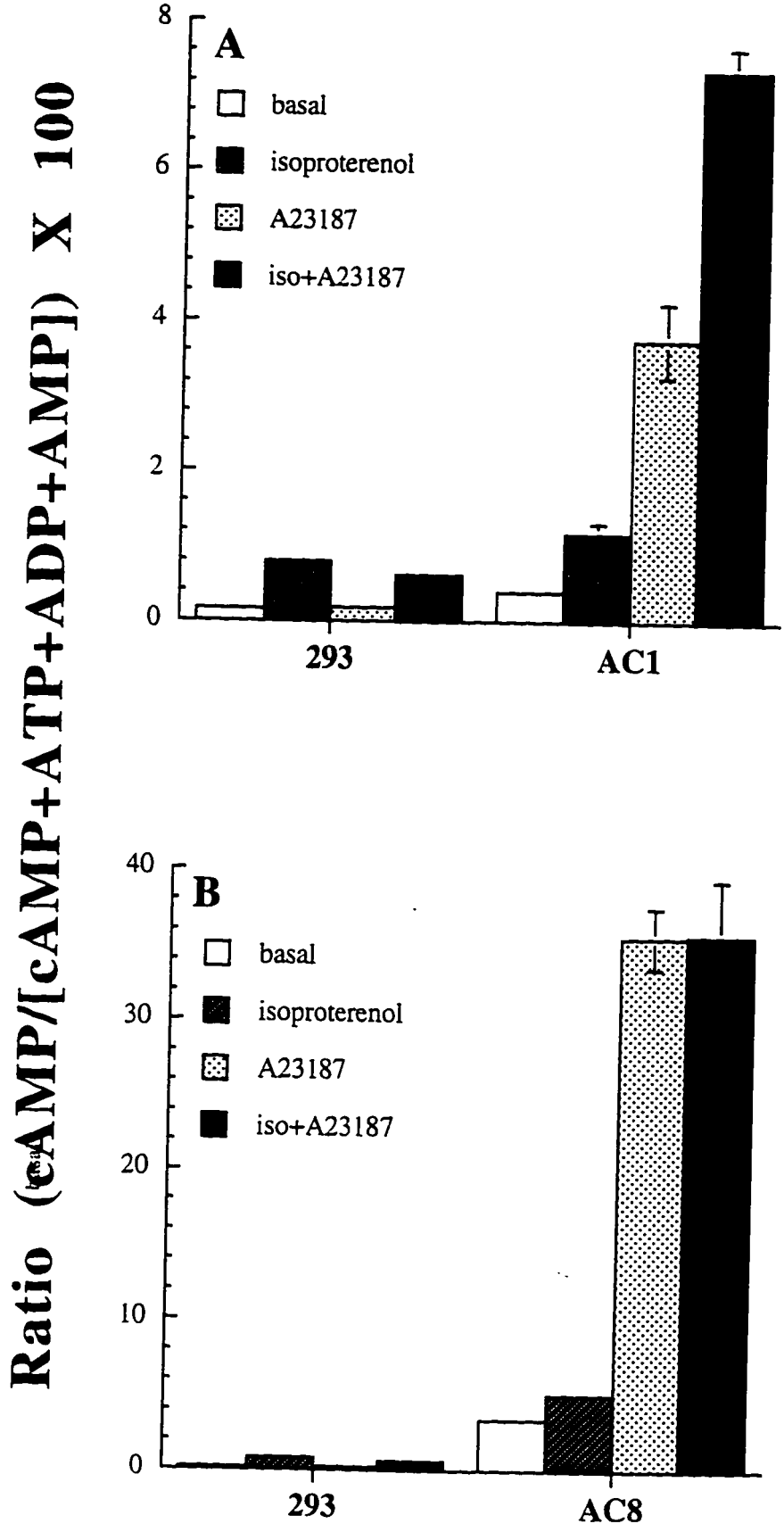

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
basal
isoproterenol
A23187
iso+A23187
8
6
4
2
0
293
AC1
B
basal
isoproterenol
A23187
iso+A23187
40
30
20
10
0
293
AC8

Figure 15. Somatostatin inhibited Ca^{2+}-stimulated cAMP levels in primary cultures of rat cortical or hippocampal neurons. Primary neuron cultures were prepared as described in Materials and Methods. After 9 days in culture, neurons were labelled overnight and then treated with 1μM A23187 in the presence or absence of 1μM somatostatin. Relative cAMP accumulation was determined as described in Materials and Methods. The data are the mean +/-SD of triplicate assays.

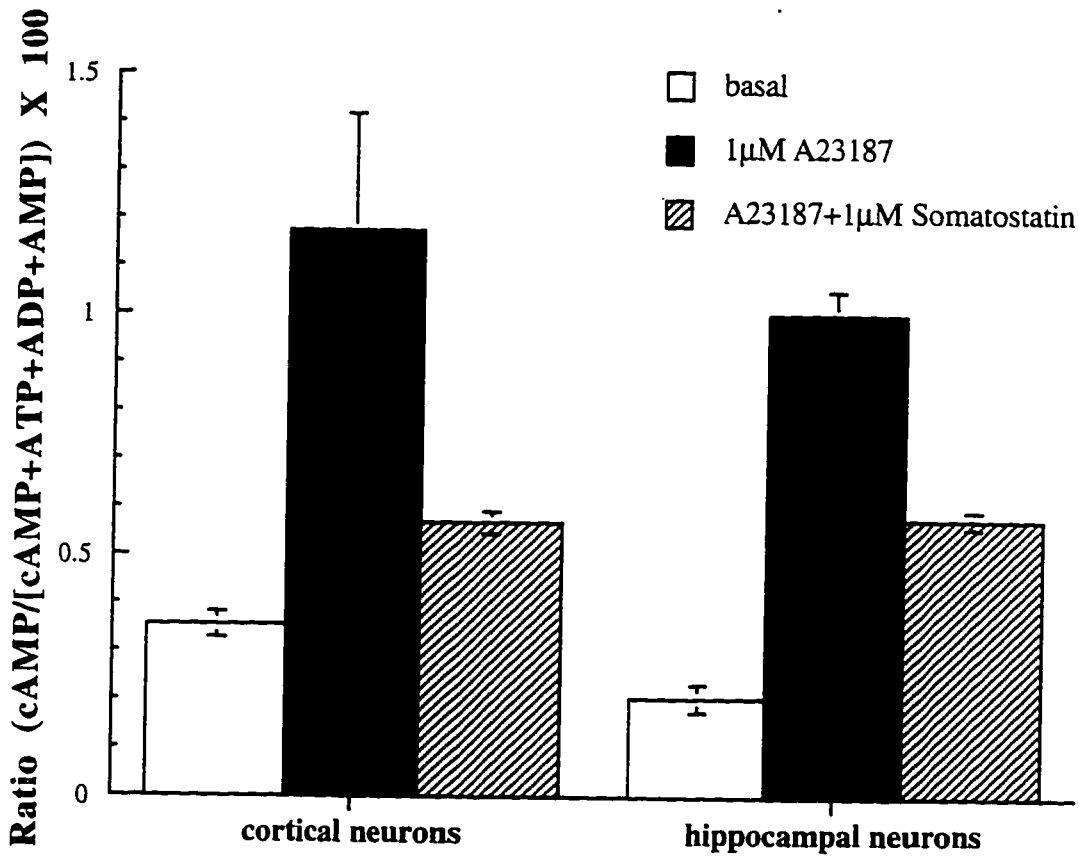

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
1.5
1
0.5
0
basal
1μM A23187
A23187+1μM Somatostatin
cortical neurons
hippocampal neurons

Table III

A Comparison of the Regulatory Properties of AC1 and AC8

Parameter	AC1	AC8
stimulation by Ca^{2+}/CaM	yes	yes
Ca^{2+}, EC_{50}	~150 nM	~750 nM
stimulation by $G_{s\alpha}$-GTPγS *in vitro*	yes	yes
stimulation by G_s *in vivo*	no	no
G_s&Ca^{2+}/CaM synergism	yes	no
stimulation by PKC	yes	ND
inhibition by $G_{i\alpha}$-GTPγS *in vitro*	yes	ND
inhibition by G_i *in vivo*	yes	no
inhibition by CaM kinase IV	yes	no
βγ sensitivity *in vitro*	inhibition	ND
βγ sensitivity *in vivo*	no	ND

ND - not determined

CHAPTER III. STIMULATION OF THE TYPE 1 AND TYPE 8 CALCIUM/ CALMODULIN-STIMULATED ADENYLYL CYCLASES BY Gs-COUPLED 5HT7 SEROTONIN RECEPTORS

Introduction

Serotonin (5-hydroxytryptamine, 5HT) is a ubiquitous neurotransmitter/ neuromodulator which elicits a wide variety of physiological events both peripherally and centrally (Graeff et al., 1996; Martin, 1994; Simansky, 1996). A large family of plasma membrane receptors bind 5HT and mediate its cellular effects (Hoyer et al., 1994). Except for $5HT_3$, 5HT receptors belong to the superfamily of G protein-coupled receptors. Among these, the recently cloned $5HT_6$ (Kohen et al., 1996; Monsma et al., 1993) and $5HT_7$ (Bard et al., 1993; Shen et al., 1993) receptors activate adenylyl cyclase(s) most likely via the heterotrimeric stimulatory G protein G_s, as expression of these receptors in cultured cells increases cAMP levels (Monsma et al., 1993; Shen et al., 1993). $5HT_6$ and $5HT_7$ each possess high affinities for a variety of antipsychotic and antidepressant drugs including clozapine, amoxapine, and amitryptiline (Bard et al., 1993; Kohen et al., 1996; Monsma et al., 1993; Shen et al., 1993), suggesting a role for these receptors in cognitive function. The CNS localization of $5HT_6$ (hippocampus, nucleus accumbens, striatum, limbic regions) (Ward et al., 1995) and $5HT_7$ (hypothalamus, hippocampus, cortex) (Shen et al., 1993) further supports a role for these receptors in mood and affect (Beeckmans and Michiels, 1996; Soares and Mann, 1997).

Although a direct role for cAMP in affective disorders has not been elucidated, there is adequate potential for at least some involvement since many clinically useful psychotherapeutic agents interfere with components of the cAMP signalling pathway (Nibuya et al., 1996). To date, there are at least nine distinct cDNA clones for mammalian

adenylyl cyclases (Bakalyar and Reed, 1990; Cali et al., 1994; Feinstein et al., 1991; Gao and Gilman, 1991; Ishikawa et al., 1992; Katsushika et al., 1992; Krupinski et al., 1989; Premont et al., 1992; Premont et al., 1996; Watson et al., 1994), the enzyme which synthesizes cAMP. Each isoform has a unique tissue distribution and differential regulatory properties (Cooper et al., 1995; Sunahara et al., 1996; Taussig and Gilman, 1995). Given that 5HT$_6$ and 5HT7 receptors couple to Gs, we felt that it would be beneficial to determine the coupling of 5HT$_6$ and 5HT7 to individual adenylyl cyclases expressed in heterologous systems, as this affords a better understanding of serotonergic control of cAMP levels in brain regions where a specific receptor-effector coupling is postulated to exist (e.g., 5HT$_6$ and AC1 in hippocampus and cerebellum (Ward et al., 1995; Xia et al., 1991); 5HT$_6$ and AC5 in striatum/nucleus accumbens (Mons and Cooper, 1994; Ward et al., 1995); 5HT7 and AC8 in the hypothalamus (Cali et al., 1994; Shen et al., 1993). In addition, it is often difficult to isolate the effects of a serotonin receptor on cAMP levels in native tissue since there are a large number of serotonin receptors, as well as a lack of specific drugs for several of these receptor subtypes (Hoyer et al., 1994).

Results detailed in this chapter indicate that 5HT$_6$ behaves as a "typical" G_S-coupled receptor, stimulating AC5, but not AC1 or AC8. On the other hand, 5HT7 stimulated both the G_S-sensitive AC5 and G_S-insensitive isoforms (AC1 and AC8) of adenylyl cyclase. Additionally, we demonstrated that 5HT7 stimulation of AC1 and AC8 is largely due to increases in intracellular Ca^{2+}, suggesting that 5HT7 is capable of coupling to a signalling pathway other than Gs. We feel that these observations enhance our understanding of serotonergic control of the cAMP and Ca2+ signalling pathways in specific regions of the CNS, as well as highlighting the possibility that adenylyl cyclases may be involved in specific neural processes (i.e., the fact that AC5 couples with 5HT$_6$, a target for psychotherapeutic drugs in striatum/nucleus accumbens, suggests a role for AC5 in cognitive function).

Materials and Methods

Materials- 3-isobutyl-1-methylxanthine, carbachol, and 5HT were from Sigma (St. Louis, MO). Clozapine and methiothepin were from Research Biochemicals, Inc. (Natick, MA). Thapsigargin, BAPTA-AM and Fura-2 AM were from Calbiochem (La Jolla, CA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). The Klenow fragment of DNA polymerase was obtained from Boehringer Mannheim (Indianapolis, IN).

Cell Culture- Human embryonic kidney 293 cells (HEK 293) were grown at 37°C in HEPES-buffered Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% bovine calf serum (BCS) in a humidified 95% air, 5% CO_2 incubator. Cell culture materials were from Life Technologies, Inc. (Gaithersburg, MD) unless otherwise indicated.

Subcloning of 5HT6 and 5HT7 cDNA fragments into pcDNAIII- The human $5HT_6$ cDNA clone in pBluescript KS- was digested with XbaI and the sticky end was filled-in with Klenow fragment. Subsequently, the $5HT_6$ cDNA was released from pBluescript KS- by digestion with XhoI. The isolated cDNA fragment was ligated into pcDNAIII (Invitrogen, San Diego, CA) which had been previously digested with EcoRV and XhoI. The correct pcDNAIII-$5HT_6$ construct was confirmed by restriction endonuclease digestion and agarose gel electrophoresis. The human $5HT_7$ cDNA clone in pCDSRα was released by digestion with EcoRI and XbaI and ligated into pcDNAIII previously digested with the same two enzymes. Positive ligations were confirmed as described above.

Coexpression of 5HT6 or 5HT7 with adenylyl cyclases in HEK 293 cells- pcDNAIII-5HT6 and pcDNAIII-5HT7 were generated as described above. The AC1 cDNA clone was isolated from a bovine brain cDNA library as previously described (Xia et al., 1991). The cDNA clone for AC5 was generously provided by Dr. Ravi Iyengar (Mount Sinai School of Medicine, New York, NY). The cDNA clone for AC8 was a gift from Dr. John Krupinski (Weis Center for Research, Geisinger Clinic, Danville, PA). Polyclonal populations of G418 (Calbiochem, 500μg/ml)- and hygromycin (Calbiochem, 500units/ml)-resistant HEK 293 cells were obtained by stable transfection using the calcium phosphate method (Chen and Okayama, 1987) of either 5HT6 or 5HT7 with either the pCEP4 expression vector or one of the aforementioned adenylyl cyclases in pCEP4. Expression of adenylyl cyclases was confirmed by cAMP accumulation assays as described below. Expression of 5HT6 and 5HT7 receptors was confirmed by cAMP accumulation assays (see below).

cAMP Accumulation Assay- Changes in intracellular cAMP levels were measured by determining the ratio of [^{3}H]-cAMP to a total ATP, ADP, and AMP pool in [^{3}H]-adenine-loaded cells as previously described (Wong et al., 1991). This assay system allows for rapid and sensitive determination of relative changes in intracellular cAMP levels. While the ratios measured between assays may show some variation, the relative changes in cAMP levels between assays is quite reproducible. Briefly, as cells in 12-well plates approached confluency (~90%), they were incubated in H-DMEM+10% BCS containing 1-2μCi/well [^{3}H]-adenine (ICN, Costa Mesa, CA) for 16-20 hours. The next day, cells were aspirated, washed once with 150mM NaCl and incubated in H-DMEM+1% pen/strep containing the indicated effectors (e.g., serotonin) plus 1mM 3-isobutyl-1-methylxanthine (IBMX) for 30 minutes. For assays during which receptor antagonists were used, cells were pretreated

with the indicated antagonists for 15 minutes in H-DMEM. Assays were initiated by addition of stimulators and IBMX in the continued presence or absence of antagonists. For assays in which "Ca^{2+}-free" conditions were used, DMEM/F-12 (1:1) lacking $CaCl_2$ but containing 0.5mM EGTA was used during both the thapsigargin pretreatment and the assay. For "normal" Ca^{2+} data points in the same assays, $CaCl_2$ was added back to 1.8mM for the pretreatment and assay. For BAPTA-AM treatments, cells were pretreated for 15 minutes with DMSO vehicle or BAPTA, and assayed as described. Longer pretreatment times tended to be toxic to the cells. Reactions were terminated by aspiration and addition of 1 ml ice-cold 5% trichloroacetic acid/1μM cAMP. Culture dishes were maintained at 4°C for 1-4 hours and acid-soluble nucleotides were separated by sequential Dowex AG50WX-4 and neutral alumina chromatography as previously described (Salomon et al., 1974). Reported data are the averages of triplicate determinations.

Results

Expression of 5HT6 and 5HT7 receptors in HEK 293 cells- Polyclonal populations of G418- and hygromycin-resistant HEK 293 cells were obtained by stable transfection of either 5HT6 or 5HT7 with pCEP4. Expression of functional receptors was determined by intracellular cAMP accumulation assays on 5HT-treated cells. HEK 293 cells do not express endogenous 5HT receptors coupled to adenylyl cyclases (data not shown). However, in cells expressing 5HT6 or 5HT7, treatment of cells with 5HT produced substantial increases in intracellular cAMP (Figures 16A and 16B, respectively). To determine if the expressed receptors demonstrated the expected pharmacology, we carried out cAMP accumulation assays over a range of 5HT concentrations in the presence or absence of several relevant antagonists. In 5HT6-expressing cells, half-maximal

stimulation of cAMP occurred at ~30nM 5HT, with maximal stimulation of ~20-fold occurring at 10μM 5HT (Figure 16A). Clozapine and methiothepin, antagonists at the $5HT_6$ receptor, shifted the 5HT activation curve to the right, yielding apparent k_i values of 35.7nM and 7.1 nM respectively. In HEK 293 cells expressing $5HT_7$, half-maximal stimulation of cAMP levels occurred at ~15nM 5HT, with maximal stimulation of ~7-fold at ~ 250nM 5HT (Figure 16B). Clozapine, chlorpromazine, and amitryptiline antagonists at $5HT_7$, shifted the 5HT activation curve to the right with apparent k_i values of 11.8nM, 137nM, and 250nM, respectively, in reasonable agreement with published values (Bard et al., 1993; Roth et al., 1994; Shen et al., 1993).

Stimulation of AC5 by 5HT6 and 5HT7 Receptors- Coupling of $5HT_6$ and $5HT_7$ to specific isoforms of adenylyl cyclase was assessed by measuring intracelluar cAMP accumulation in polyclonal populations of HEK 293 cells coexpressing $5HT_6$ or $5HT_7$ with a specific isoform of adenylyl cyclase. Cells coexpressing $5HT_6$ and AC5 demonstrated robust increases in cAMP levels by 5HT (Figure 17A). AC5 was stimulated approximately 7-fold by 10μM 5HT. Similarly, in cells coexpressing $5HT_7$ and AC5, cAMP levels were elevated ~7-fold by 10μM 5HT (Figure 17B). Pharmacologically, the response of AC5 to 5HT in both $5HT_6$/AC5 and $5HT_7$/AC5 cells was blocked by clozapine and methiothepin (Figure 18). These results indicate that $5HT_6$ and $5HT_7$ couple to AC5, probably through G_S.

Lack of Coupling of 5HT6 to AC1 and AC8- AC1 is not stimulated by G_S-coupled receptors *in vivo* unless intracellular Ca^{2+} is simultaneously elevated (Impey et al., 1994; Wayman et al., 1994). AC8 is not stimulated by G_S-coupled receptors under any conditions tested to date (Cali et al., 1994)(and see Chapter 2). To determine if $5HT_6$ was

capable of stimulating AC1 or AC8, cells were treated with varying concentrations of 5HT and intracellular cAMP was measured. $5HT_6$ did not stimulate either AC1 or AC8 at any concentrations of 5HT tested (Figure 19), although in the same experiments, cells expressing $5HT_6$ with pCEP4 showed robust increases in cAMP (Figure 19). In fact, correction of the cAMP ratios by subtracting the endogenous activity from AC1 or AC8 ratios yielded negative values. In these same cells, the Ca^{2+} ionophore A23187 robustly stimulated cAMP levels (data not shown). These data combined with that from AC5-containing cell lines indicate that $5HT_6$ behaves as a typical G_S-coupled receptor in that it stimulates AC5, but it does not stimulate the Ca^{2+}-stimulated adenylyl cyclases AC1 and AC8.

$5HT_7$ stimulates AC1 and AC8- To determine whether cells coexpressing $5HT_7$ with either AC1 or AC8 responded to 5HT with increases in cAMP, cells were treated with several concentrations of 5HT and cAMP accumulation was measured. Unexpectedly, $5HT_7$ stimulated both AC1 and AC8 (Figures 20A and 20B, respectively) without any additional experimental manipulations (e.g., the presence of Ca^{2+} ionophores). 1μM 5HT stimulated AC1 ~6-fold and AC8 ~4-fold. Activation of AC1 and AC8 by $5HT_7$ was blocked by both clozapine and methiothepin, indicating that the stimulation was receptor-mediated (Figures 20A and 20B).

$5HT_7$ stimulation of AC1 and AC8 is mediated by increases in intracellular Ca^{2+}- Since AC1 and AC8 are not activated by G_S-coupled receptors *in vivo* (Cali et al., 1994; Impey et al., 1994; Wayman et al., 1994) (and see Chapter 2), our unexpected finding that the "G_S-coupled" $5HT_7$ receptor stimulated both AC1 and AC8 prompted us to determine if intracellular Ca^{2+} played a role in this phenomenon. To assess the role of intracellular

Ca^{2+} increases in 5HT7 stimulation of AC1 and AC8, we carried out intracellular cAMP accumulation assays under "normal" Ca^{2+}-containing conditions (1.8mM extracellular $CaCl_2$) or "Ca^{2+}-free" conditions in which cells were pretreated for 30 minutes with 250nM thapsigargin in Ca^{2+}-free media containing 0.5mM EGTA. This thapsigargin treatment has been previously shown to release intracellular Ca^{2+} stores (Wayman et al., 1995). Following pretreatment, cells were aspirated and assayed in the same medium (minus thapsigargin) with either 0.5mM EGTA (Ca^{2+}-free) or 1.8mM $CaCl_2$ (normal Ca^{2+}) present. Under "normal" Ca^{2+} conditions, 5HT7 stimulated AC1 quite robustly and AC8 moderately (Figures 21A and 21B). Under conditions in which internal Ca^{2+} stores were depleted and extracellular Ca^{2+} was absent, 5HT7 stimulation of AC1 was attenuated 40-50% (Figure 21A), while AC8 stimulation was almost completely abolished (Figure 21B). The residual stimulation of AC1 by 5HT may be due to incomplete removal of all Ca^{2+}, thus, rendering the enzyme slightly G_S-sensitive (Wayman et al., 1994).

A second method of determining if 5HT7 activation of the Ca^{2+}-stimulated adenylyl cyclases was due to 5HT-induced increases of intracellular Ca^{2+} is to carry out intracellular cAMP assays in which the cells were loaded with the Ca^{2+} chelator BAPTA-AM prior to assay. BAPTA- AM had no substantial effect on cAMP accumulation in cell expressing 5HT7 alone (data not shown). However, BAPTA-AM pretreatment attenuated 5HT7 stimulation of AC1 by 50-70% depending upon the concentration of 5HT used (Figure 22A), while the stimulation of AC8 by 5HT7 was completely blocked by BAPTA-AM (Figure 22B), similar to the thapsigargin depletion experiments. The incomplete block of 5HT stimulation of AC1 by BAPTA-AM was likely due to the difficulty of obtaining high enough intracellular concentrations of BAPTA-AM to chelate all of the Ca^{2+} without causing toxicity to the cells. Since AC8 has a lower Ca^{2+} sensitivity than AC1 (Villacres et al., 1995), the Ca^{2+} depletion/chelation paradigms may remove enough Ca^{2+} to render

AC8 insensitive to the remaining low levels of Ca^{2+}, while AC1 still retains sensitivity. At any rate, these results indicate that 5HT7 stimulation of AC1 and AC8 is largely due to increases in intracellular Ca^{2+}, a novel, unexpected finding.

Discussion

Serotonin is a ubiquitous hormone/neurotransmitter which modulates a variety of physiological functions, including sleep (Leonard, 1996), appetite (Simansky, 1996), vascular tone (Martin, 1994), sexual behavior (Meston and Gorzalka, 1992), and mood (Graeff et al., 1996). The increasingly large family of serotonin receptors (Hoyer et al., 1994) has probably evolved in order to carry out the diverse roles of serotonin. Several of the recently cloned members of the serotonin receptor family (i.e., $5HT_6$ and 5HT7) are coupled to stimulation of adenylyl cyclase, and in brain, their mRNA expression patterns overlap with those of several adenylyl cyclases. To better understand serotonergic control of cAMP levels in regions of brain where a specific receptor/effector coupling may exist, we have coexpressed $5HT_6$ or 5HT7 with either AC1, AC5, or AC8 in HEK 293 cells to evaluate coupling between $5HT_6$ or 5HT7 and specific adenylyl cyclases. Our data demonstrate that $5HT_6$ behaved as a typical G_S-coupled receptor in that it stimulated AC5, but not AC1 or AC8, which have previously shown to be insensitive to G_S-coupled receptors (Cali et al., 1994; Impey et al., 1994; Wayman et al., 1994). Unexpectedly, 5HT7 not only stimulated AC5, but also AC1 and AC8. Since AC1 and AC8 are insensitive to G_S-coupled receptors (Cali et al., 1994; Impey et al., 1994; Wayman et al., 1994), we tested the role of Ca^{2+} in 5HT7 stimulation of AC1 and AC8. To our surprise, we found that 5HT7, but not $5HT_6$ was able to stimulate the Ca^{2+}-sensitive adenylyl cyclases, most likely via increases of intracellular Ca^{2+}. Depletion with thapsigargin in

Ca^{2+}-free media or chelation of Ca^{2+} with BAPTA-AM attenuated 5HT7 stimulation of AC1 and abolished 5HT7 of AC8. Therefore, 5HT7 stimulation of the Ca^{2+}/CaM-stimulated adenylyl cyclases AC1 and AC8 was likely due to elevations of intracellular Ca^{2+}. The remaining stimulation of AC1 may be due an incomplete block of Ca^{2+} elevations, thus allowing a response to G_S and Ca^{2+} described by Wayman et al (Wayman et al., 1994) in which Ca^{2+}/CaM bound to AC1 sensitizes the enzyme to G_S stimulation. The complete inhibition of AC8 stimulation by 5HT7 may be reflective of its substantially lower Ca^{2+} sensitivity than AC1(Villacres et al., 1995), and thus, any residual Ca^{2+} is too low to stimulate AC8. Although, we have not determined the mechanism for 5HT7-induced increases of intracellular Ca^{2+}, to our knowledge, the ability of 5HT7 activation to increase intracellular Ca^{2+} has not been previously reported.

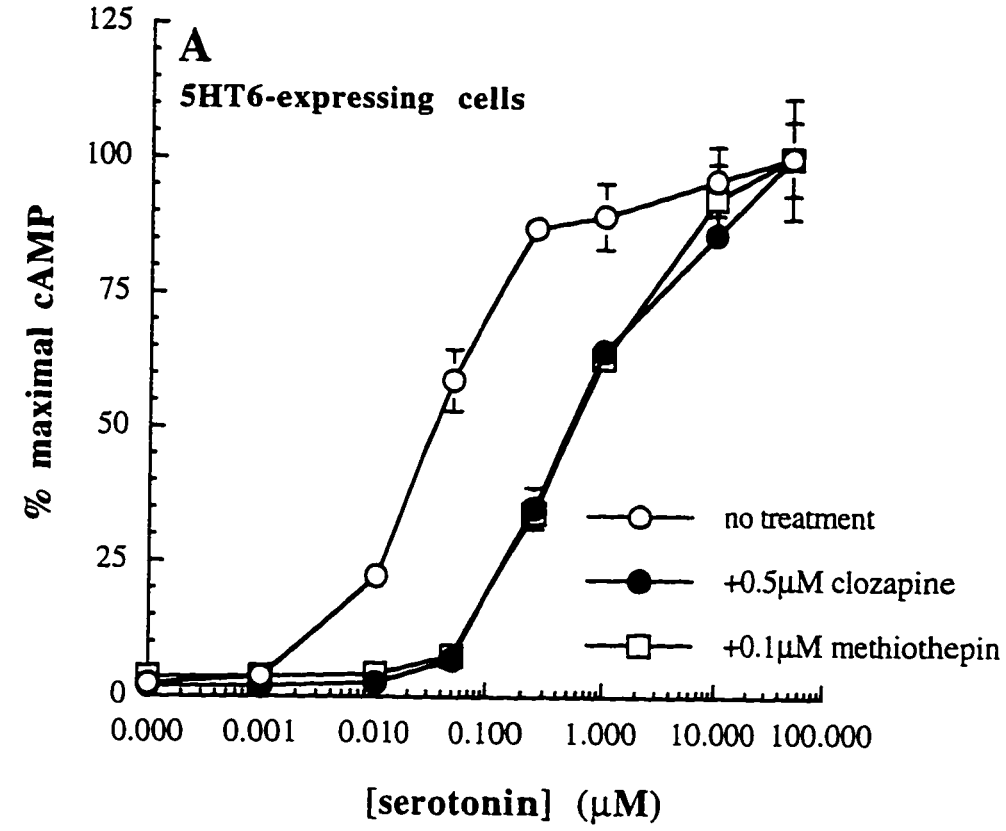
In terms of coupling, the 5HT6/AC5 coupling is interesting since both 5HT6 and AC5 are expressed in striatum/nucleus accumbens, important loci for antipsychotic drug effects (Bachus and Kleinman, 1996). This suggests that antagonism of 5HT6 stimulation of AC5 in striatum/nucleus accumbens may contribute, in part, to the therapeutic effects of clozapine and other psychotherapeutic agents. On a broader level, the coupling of 5HT6 and AC5 indicates that AC5 may mediate serotonergic effects on striatal function.

From our ppoint of view, the coupling of 5HT7 to AC1 and AC8 was most surprising. Coexpression of mRNA for 5HT6, 5HT7, AC1, AC8 occurs in hippocampus (Cali et al., 1994; Shen et al., 1993; Ward et al., 1995; Xia et al., 1991), yet, these data indicate that 5HT7, but not 5HT6, might mediate 5HT activation of AC1 or AC8 in hippocampus via increases of intracellular Ca^{2+}. Functionally, the ability of 5HT7 to stimulate AC1 and AC8 implies that this receptor may play a role in synaptic plasticity in the hippocampus. However, the role of 5HT in the hippocampus is unclear, partly because nearly all of the receptors for 5HT are expressed there, and also due to the fact that specific drugs are not yet available for each receptor subtype. Once 5HT7-selective agents are

available, it would be interesting to test whether or not activation of this receptor in neurons also increases intracellular Ca^{2+}, as well as if 5HT7 is involved in certain forms of synaptic plasticity. Finally, the coupling of 5HT7 to AC8 may also be relevant in the context of hypothalamic function (e.g., endocrine or circadian regulation).

In summary, these results demonstrate that 5HT6 acts as a typical G_S-coupled receptor by stimulating AC5, but not AC1 or AC8. On the other hand, 5HT7 stimulated AC5, as well as AC1 and AC8. The stimulation of the latter two was due, in large part, to increases of intracellular Ca^{2+}, an unexpected result. In the future, it will be of interest to elucidate the mechanism for 5HT7 increases of intracellular Ca^{2+}, as well as utilizing specific 5HT6 and 5HT7 antagonists, when they become available, to probe the regulation of cAMP by these receptors in specific areas of brain and other tissues.

Figure 16. Expression of $5HT_6$ and $5HT_7$ receptors in HEK 293 cells. A, HEK 293 cells expressing $5HT_6$ were pretreated for 15 minutes with 0.5μM clozapine, 0.1μM methiothepin. or vehicle (DMSO). Assays were initiated by the addition of the indicated concentrations of 5-HT and IBMX in the continued presence or absence of antagonist. B, HEK 293 cells expressing $5HT_7$ were pretreated for 15 minutes with 0.5μM clozapine, 1μM chlorpromazine, 1μM amitryptiline, or vehicle (DMSO). Assays were initiated by the addition of the indicated concentrations of 5HT and IBMX in the continued presence or absence of antagonist. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays. Approximate k_i values were calculated using the Schild equation.


A
5HT6-expressing cells
% maximal cAMP
125
100
75
50
25
0
0.000
0.001
0.010
0.100
1.000
10.000
100.000
[serotonin] (μM)
no treatment
+0.5μM clozapine
+0.1μM methiothepin

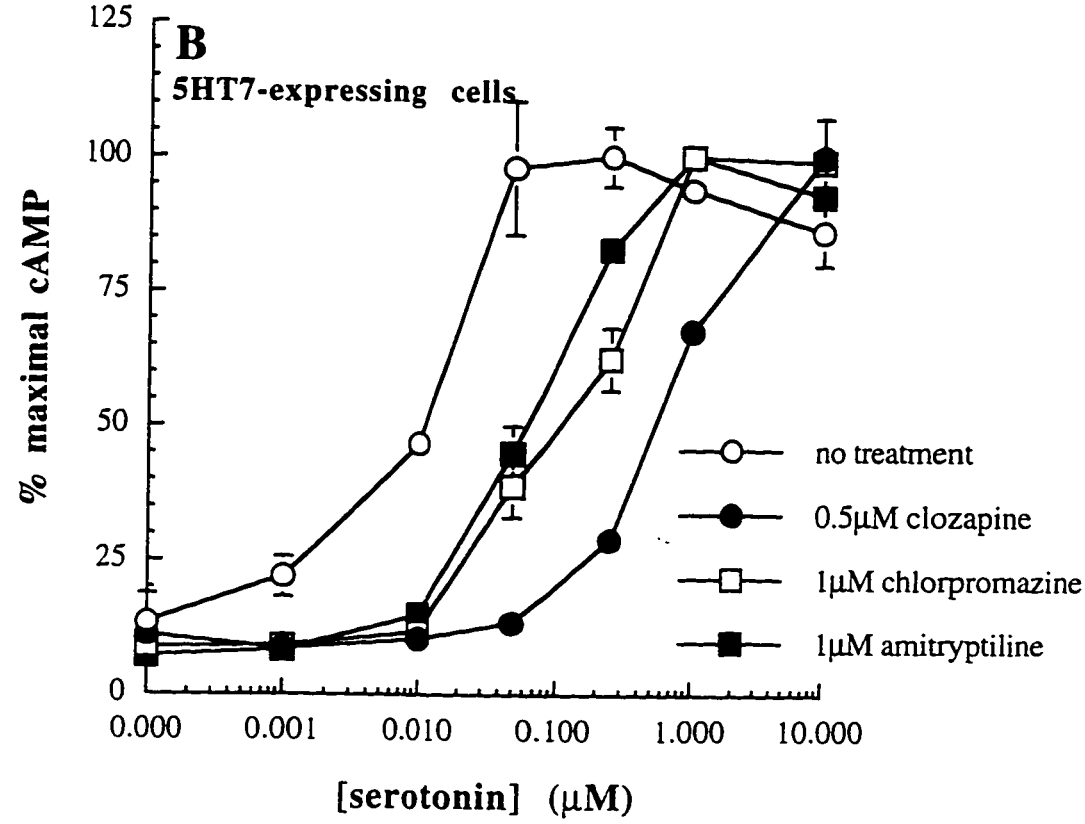

B
5HT7-expressing cells
% maximal cAMP
125
100
75
50
25
0
0.000
0.001
0.010
0.100
1.000
10.000
[serotonin] (μM)
no treatment
0.5μM clozapine
1μM chlorpromazine
1μM amitryptiline

Figure 17. Stimulation of AC5 by $5HT_6$ and $5HT_7$ receptors in HEK 293 cells. A, HEK 293 cells coexpressing $5HT_6$ and AC5 were treated with the indicated concentrations of 5HT. B, HEK 293 cells coexpressing $5HT_7$ and AC5 were treated with the indicated concentrations of 5HT. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays. The contribution of the endogenous HEK 293 cell adenylyl cyclases has been subtracted from AC5-transfected cells' cAMP values.

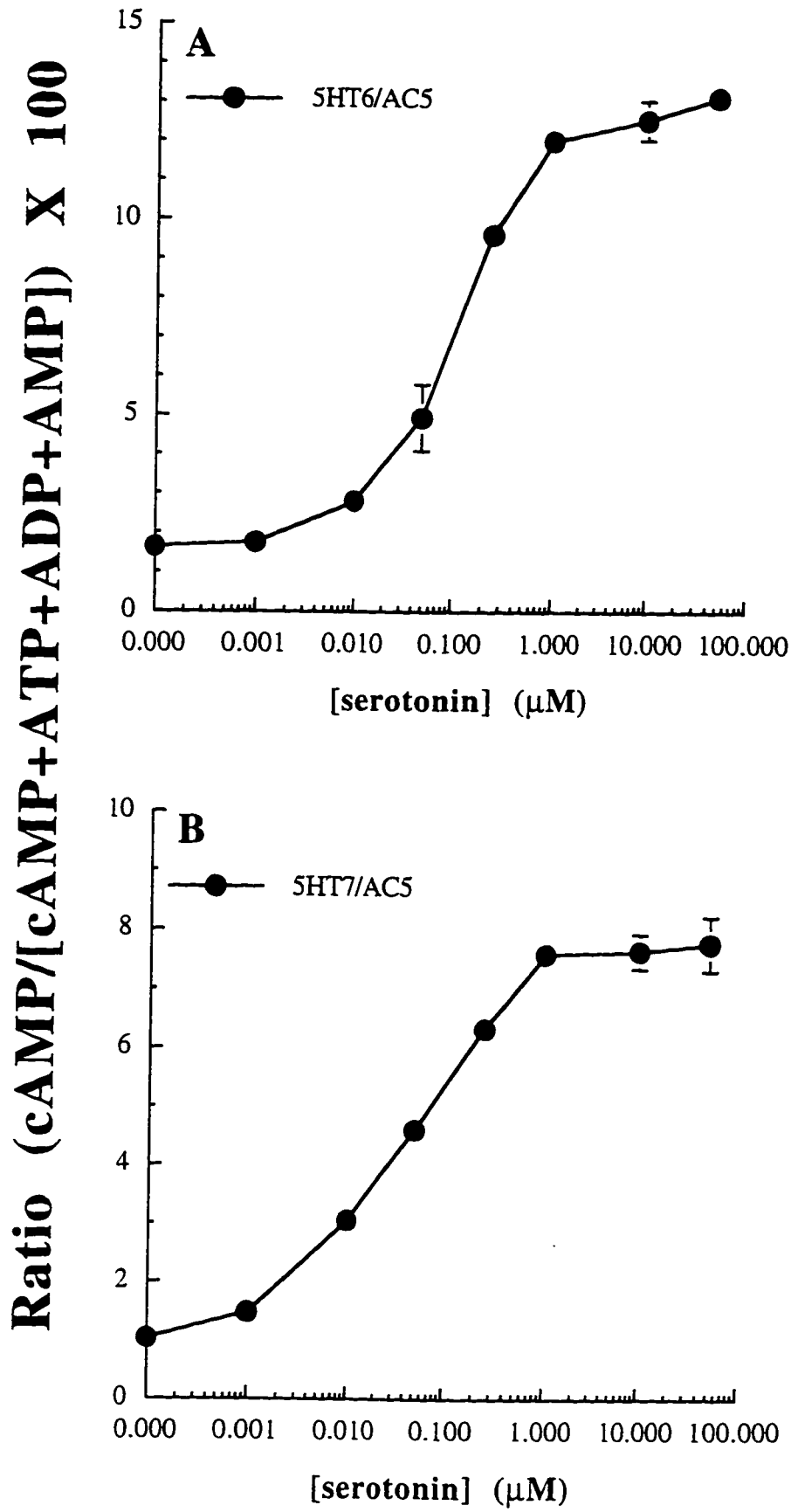

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
5HT6/AC5
15
10
5
0
0.000
0.001
0.010
0.100
1.000
10.000
100.000
[serotonin] (μM)
B
5HT7/AC5
10
8
6
4
2
0
0.000
0.001
0.010
0.100
1.000
10.000
100.000
[serotonin] (μM)

Figure 18. Stimulation of AC5 by $5HT_6$ and $5HT_7$ is blocked by clozapine and methiothepin. A, HEK 293 cells coexpressing $5HT_6$ and AC5 were pretreated with 10μM clozapine, 1μM methiothepin, or DMSO vehicle for 15 minutes. cAMP assays were initiated by addition of vehicle or 1μM 5HT in the presence or absence of the indicated antagonists. B, HEK 293 cells coexpressing $5HT_7$ and AC5 were pretreated with 10μM clozapine, 1μM methiothepin, or DMSO vehicle for 15 minutes. cAMP assays were initiated by addition of vehicle or 1μM 5HT in the presence or absence of the indicated antagonists. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays. The contribution of the endogenous HEK 293 cell adenylyl cyclases has been subtracted from AC5-transfected cells' cAMP values.

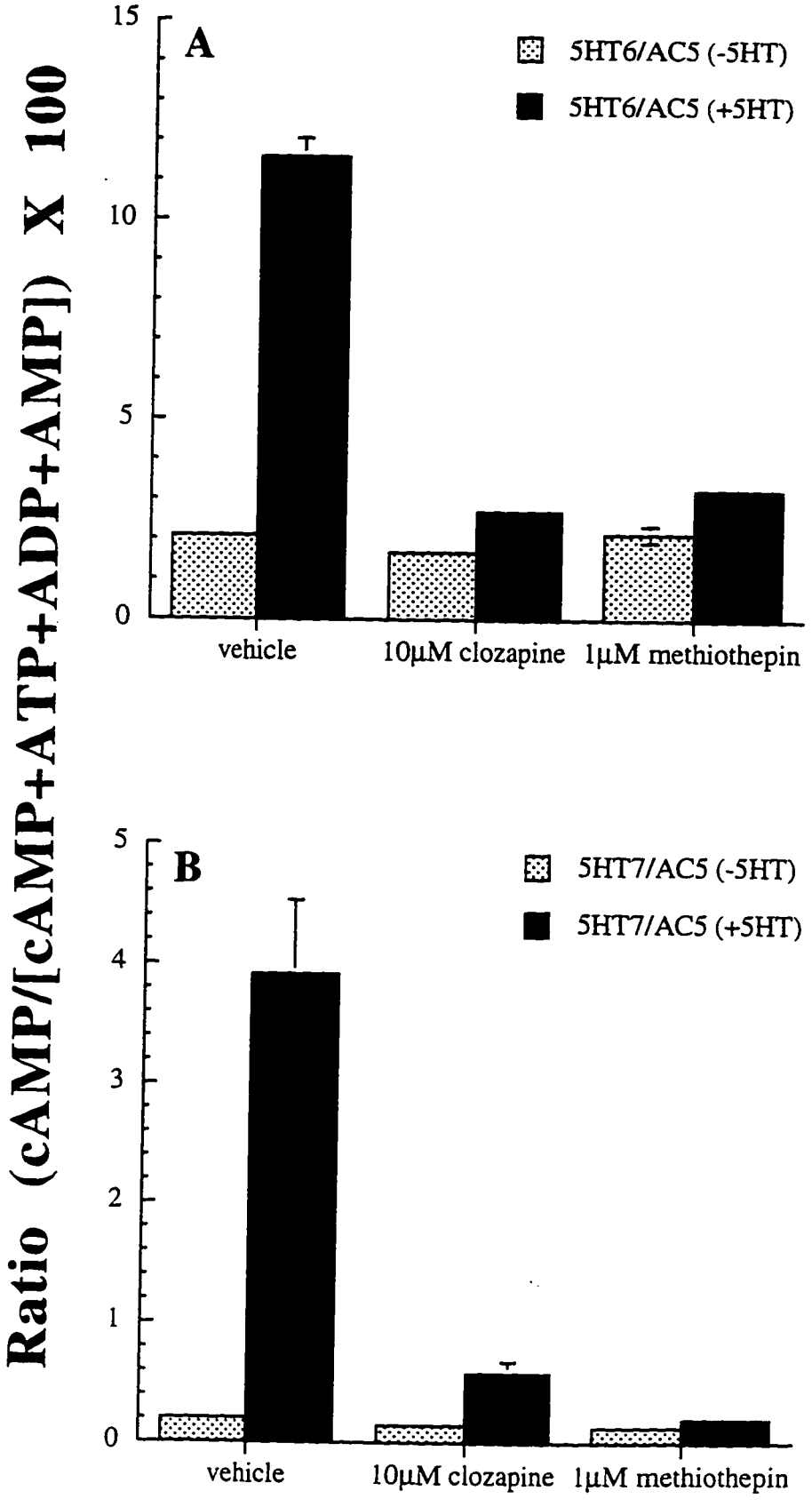

Ratio ([cAMP]/[cAMP+ATP+ADP+AMP]) X 100
A
15
10
5
0
5HT6/AC5 (-5HT)
5HT6/AC5 (+5HT)
vehicle
10μM clozapine
1μM methiothepin
B
5
4
3
2
1
0
5HT7/AC5 (-5HT)
5HT7/AC5 (+5HT)
vehicle
10μM clozapine
1μM methiothepin

Figure 19. AC1 and AC8 are not stimulated by $5HT_6$ receptors. A, HEK 293 cells coexpressing $5HT_6$ and AC1 were treated with the indicated concentrations of 5HT. B, HEK 293 cells coexpressing $5HT_6$ and AC8 were treated with the indicated concentrations of 5HT. C, HEK 293 cells expressing $5HT_6$ and pCEP4 vector were treated with the indicated concentrations of 5HT. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays.

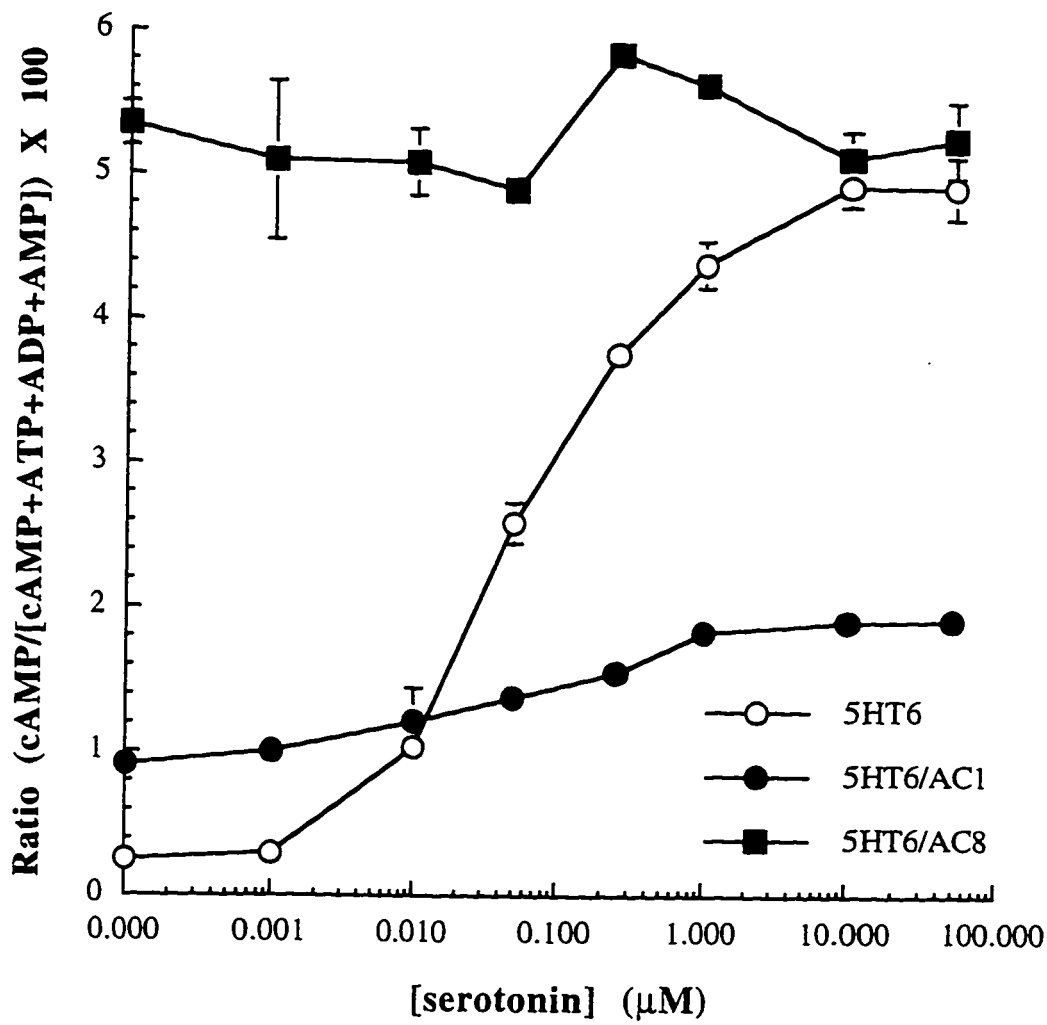

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
6
5
4
3
2
1
0
0.000
0.001
0.010
0.100
1.000
10.000
100.000
[serotonin] (μM)
5HT6
5HT6/AC1
5HT6/AC8

Figure 20. Stimulation of AC1 and AC8 by 5HT7 receptors. A, HEK 293 coexpressing 5HT7 and AC1 (A) or 5HT7 and AC8 (B) were treated with the indicated concentrations of 5HT in the presence or absence of 10μM clozapine or 1μM methiothepin (abbreviated "meth" for space). Antagonists were added to cells 15 minutes prior to initiation of assays. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays. The contribution of the endogenous HEK 293 cell adenylyl cyclases has been subtracted from the AC1- or AC8-expressing cells' cAMP values.

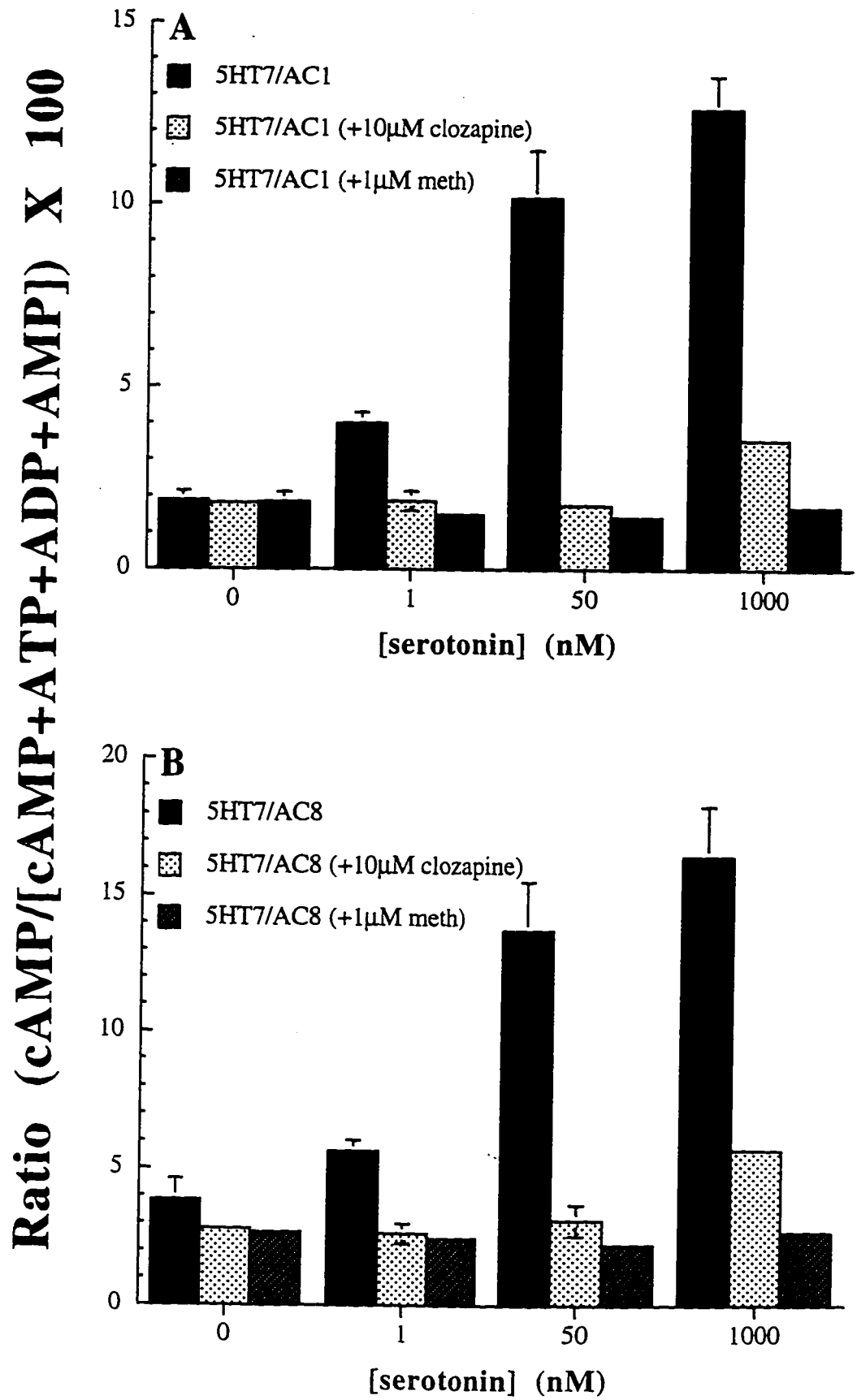

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
5HT7/AC1
5HT7/AC1 (+10μM clozapine)
5HT7/AC1 (+1μM meth)
15
10
5
0
0
1
50
1000
[serotonin] (nM)
B
5HT7/AC8
5HT7/AC8 (+10μM clozapine)
5HT7/AC8 (+1μM meth)
20
15
10
5
0
0
1
50
1000
[serotonin] (nM)

Figure 21. Intracellular Ca^{2+} depletion attenuates 5HT7 stimulation of AC1 and AC8. HEK 293 cells expressing 5HT7 (A), 5HT7 and AC1 (B), or 5HT7 and AC8 (C) were pretreated for 30 minutes with DMSO vehicle in normal DMEM/F-12 (1:1) ("+Ca^{2+} conditions") or 250nM thapsigargin in Ca^{2+}-free DMEM/F-12 (1:1) supplemented with 0.5mM EGTA ("-Ca^{2+} conditions). Following pretreatment, the media was aspirated and cells were treated with the indicated concentrations of 5HT and assayed for cAMP accumulation under the same conditions as above (i.e., +/- Ca^{2+} conditions), except that thapsigargin or DMSO was omitted. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays. The contribution of the endogenous HEK 293 cell adenylyl cyclases has been subtracted from the AC1- or AC8-expressing cells' cAMP values.

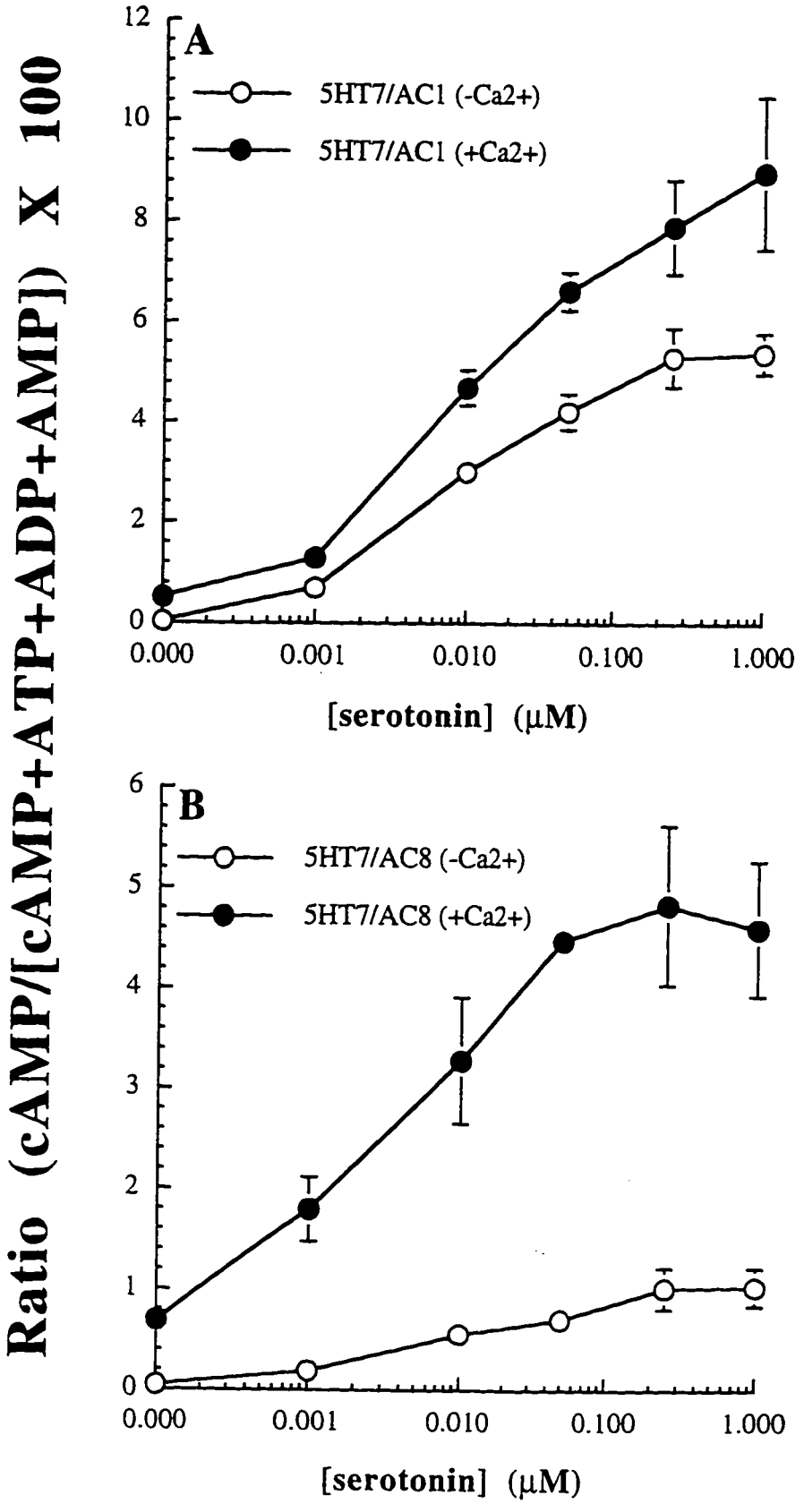
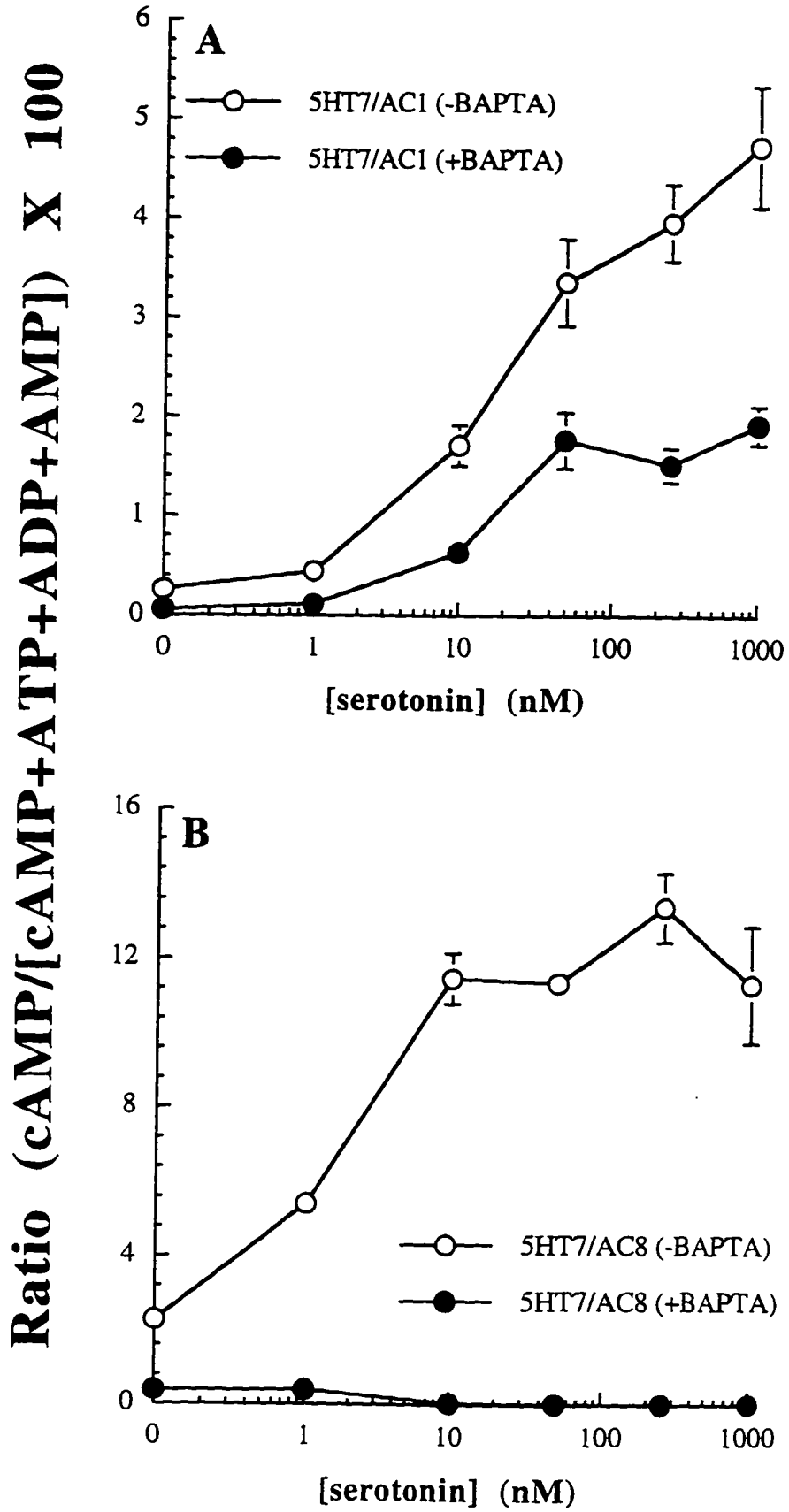

Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
A
5HT7/AC1 (-Ca2+)
5HT7/AC1 (+Ca2+)
12
10
8
6
4
2
0
0.000
0.001
0.010
0.100
1.000
[serotonin] (μM)
B
5HT7/AC8 (-Ca2+)
5HT7/AC8 (+Ca2+)
6
5
4
3
2
1
0
0.000
0.001
0.010
0.100
1.000
[serotonin] (μM)

Figure 22. Intracellular Ca^{2+} chelation inhibits 5HT7 stimulation of AC1 and AC8. HEK 293 cells expressing 5HT7 (A), 5HT7 and AC1 (B), or 5HT7 and AC8 (C) were pretreated for 15 minutes with 80μM BAPTA-AM or DMSO vehicle in H-DMEM +pen/strep. Following the preincubation period, cells were treated with the indicated concentrations of 5HT in the presence or absence of 80μM BAPTA-AM. Relative cAMP accumulation was determined as described under "Materials and Methods". The data are the mean +/- S.D. of triplicate assays.


A
5HT7/AC1 (-BAPTA)
5HT7/AC1 (+BAPTA)
Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
[serotonin] (nM)
B
5HT7/AC8 (-BAPTA)
5HT7/AC8 (+BAPTA)
[serotonin] (nM)

CHAPTER IV. *IN VIVO* REGULATION OF AC4 AND IMMUNOHISTOCHEMICAL LOCALIZATION OF THE βγ-STIMULATED ADENYLYL CYCLASES AC2 AND AC4 IN MOUSE HIPPOCAMPUS

Introduction

The regulation of adenylyl cyclase by G protein-coupled receptors is a classical mechanism which exists in most, if not all, tissues. A stimulatory G protein (G_s) couples hormones such as norepinephrine to increases in cAMP levels, while an inhibitory G protein (G_i) couples hormones (e.g., acetylcholine, somatostatin) to inhibition of adenylyl cyclase. In certain tissues such as lung (Pian and Dobbs, 1995) and brain (Andrade, 1993; Gereau IV and Conn, 1994), a G_i input paradoxically potentiates rather than inhibits the stimulatory input. In particular, in the rodent hippocampus, increases in cAMP produced by isoproterenol (a β-adrenergic agonist) are enhanced by activation of G_i-coupled 5HT1A and $GABA_B$ receptors (Andrade, 1993), as well as metabotropic glutamate receptors (Gereau IV and Conn, 1994). In addition, these treatments enhance the excitability of CA1 pyramidal cells (Andrade, 1993; Gereau IV and Conn, 1994), which may be an important functional parameter contributing to long-term changes in synaptic strength.

Although the Ca^{2+}-stimulated adenylyl cyclases (AC1 and AC8) are thought to play a role in synaptic plasticity in the hippocampus (Choi et al., 1993; Villacres et al., 1995; Weisskopf et al., 1994; Xia et al., 1995), other isoforms of adenylyl cyclase may be necessary for specific forms of synaptic plasticity. For example, at the mf-CA3 synapse, long-term potentiation (LTP), a cellular model for learning and memory, appears to be dependent on opioid neurotransmission (Williams and Johnston, 1996). Opioid receptors are coupled to the pertussis toxin-sensitive G_i/G_o class of G proteins, and, in fact, pertussis toxin prevents the development of LTP at the mf-CA3 synapse (Ito et al., 1988).

In addition, elevations of cAMP are required during mf-CA3 LTP (Huang et al., 1995; Huang et al., 1994; Weisskopf et al., 1994) as well as for L-LTP in CA1(Frey et al., 1993). Therefore, it is necessary to determine which isoforms of adenylyl cyclase are expressed in the hippocampus, including those which might mediate G_i-coupled receptor enhancement of G_s-stimulated cAMP levels.

To date, there are at least nine distinct isoforms of adenylyl cyclase (designated AC1-AC9) (Bakalyar and Reed, 1990; Cali et al., 1994; Feinstein et al., 1991; Gao and Gilman, 1991; Hellevuo et al., 1995; Ishikawa et al., 1992; Katsushika et al., 1992; Krupinski et al., 1989; Premont et al., 1992; Premont et al., 1996; Yoshimura and Cooper, 1992). Each is distributed and regulated in a unique manner (Choi et al., 1993; Cooper et al., 1995; Iyengar, 1993; Sunahara et al., 1996; Taussig and Gilman, 1995; Xia et al., 1995). mRNA has been detected for AC1 (Xia et al., 1991), AC2 (Furuyama et al., 1993), AC3 (Glatt and Snyder, 1993), AC8 (Cali et al., 1994), and AC9 (Premont et al., 1996) in the mammalian hippocampus. Interestingly, the regulatory properties of AC2 and AC4 are consistent with their playing a role in the G_i-mediated potentiation of G_s-stimulated cAMP levels in the hippocampus. $G_{s\alpha}$ stimulates each isoform and $\beta\gamma$ release, presumably from G_i, causes further activation (Federman et al., 1992; Feinstein et al., 1991; Gao and Gilman, 1991; Lustig et al., 1993).

Although AC2 has been extensively characterized in intact cells (Federman et al., 1992; Jacobowitz et al., 1993; Lustig et al., 1993; Tsu and Wong, 1996), the regulatory properties of AC4 have not been defined *in vivo*. To assess whether one or both of these adenylyl cyclase isoforms may be involved in control of cAMP in the hippocampus, we have characterized the regulation of AC4 *in vivo* in intact cells, as well as determining the immunohistochemical localization of AC2 and AC4 protein in the mouse hippocampus. We have also examined the distribution of AC4 protein in other areas of mouse brain. We

have observed that both AC2 and AC4 are expressed in mouse hippocampus. In addition, we demonstrated that, in intact cells, AC4 responds to paired G_s and G_i signals with suprastimulation. These findings suggest that the presence of AC2 and AC4 in the hippocampus may account for some of the electrophysiological and biochemical effects resulting from simultaneous coactivation of G_s-coupled and G_i-coupled receptors (Andrade, 1993; Gereau IV and Conn, 1994), as well as suggesting a possible role for AC2 and AC4 in effecting long-term changes in synaptic strength.

Materials and Methods

Materials- 3-isobutyl-1-methylxanthine (IBMX), isoproterenol, serotonin, and somatostatin-14 were from Sigma (St. Louis, MO). Pertussis toxin was obtained from List Biological (Campbell, CA). Restriction endonucleases and DNA ligase were from New England Biolabs (Beverly, MA).

Cell Culture- Human embryonic kidney 293 (HEK 293) cells were grown at 37°C in HEPES-buffered Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% bovine calf serum (BCS) in a humidified 95% air/5% CO_2 incubator. Cell culture materials were from Life Technologies, Inc. (Gaithersburg, MD) unless otherwise indicated.

Expression of the 5HT7 receptor and/or AC4 in HEK 293 Cells- The AC4 cDNA clone was generously provided by Dr. Wei-Jen Tang and Dr. Alfred G. Gilman (University of Texas Southwestern Medical Center, Dallas, TX). The AC4 insert was released from pBluescript (Stratagene, La Jolla, CA) by digestion with KpnI and BamHI and ligated into

the pCEP4 expression vector (Invitrogen, San Diego, CA). The 5HT7 receptor cDNA clone was a gift from Dr. Mark Hamblin (VA Medical Center, Seattle, WA). It was ligated into pcDNAIII (Invitrogen, San Diego, CA). Polyclonal populations of G418 (Calbiochem, La Jolla, CA) and/or hygromycin (Calbiochem, La Jolla, CA; 500units/ml)-resistant 293 cells were obtained by stable transfection of the pcDNAIII or pCEP4 expression vector and/or pcDNAIII-5HT7 or pCEP4-AC4 using the calcium phosphate method (Chen and Okayama, 1987). All stable cell lines were created from the same parental population of HEK 293 cells. Expression of 5HT7 or transfected adenylyl cyclases was determined by cAMP accumulation assays as described below.

cAMP Accumulation Assay- Changes in intracellular cAMP levels were measured by determining the ratio of [^{3}H]-cAMP to a total ATP, ADP, and AMP pool in [^{3}H]-adenine-loaded cells as described (Wong et al., 1991). This assay system allows for rapid and sensitive determination of relative changes in intracellular cAMP levels. While the ratios measured between assays may show some variation, the relative changes in cAMP levels between assays is quite reproducible. Briefly, as cells in 12-well plates approached confluency (~90%), they were incubated in H-DMEM+10% BCS containing 2μCi/well [^{3}H]-adenine (ICN, Costa Mesa, CA) for 16-20 hours. The next day, cells were aspirated, washed once with 150mM NaCl and incubated in H-DMEM+1% pen/strep containing the indicated effectors (e.g., isoproterenol, serotonin, somatostatin) plus 1mM IBMX for 30 minutes. Reactions were terminated by aspiration and addition of 1 ml ice-cold 5% trichloroacetic acid/1μM cAMP. Culture dishes were maintained at 4^{o}C for 1-4 hours and acid-soluble nucleotides were separated by sequential Dowex AG50WX-4 and neutral alumina chromatography as described. Reported data are the averages of triplicate

determinations. Pertussis toxin (List Biological, Campbell, CA), when used, was added to cells along with [^{3}H]-adenine for 16-20 hours.

Transient Coexpression of AC4 with the Carboxy-terminus of β-Adrenergic Receptor Kinase 1 in HEK 293 cells-The peptide minigene construct encoding the carboxy-terminus of β-adrenergic receptor kinase 1 (βARK1-ct) in the pRK5 plasmid was generously provided by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). The G_S-coupled serotonin receptor 5-HT7 cDNA was a gift from Dr. Mark Hamblin (VA Medical Center, Seattle, WA). Briefly, the night before transfection, cells were plated in 100mm plates at 70% density. The following morning, each plate was transfected with 8μg total DNA (1μg 5-HT7, 2.5μg pCEP4 or pCEP4-AC4, 4μg pcDNAIII (Invitrogen, San Diego, CA),or pRK-βARK1-ct, and 0.5μg RSV-β-galactosidase) in H-DMEM in the presence of 50-60μl of Lipofectamine (Life Technologies, Inc., Gaithersburg, MD). After 5 hours, cells were rinsed with H-DMEM+1% pen/strep+10% BCS and maintained for 24 hours under normal conditions. The following day, cells were split, pooled by transfection, and seeded into 12-well culture dishes (1 transfected plate/12-well dish) for cAMP assays as well as into 12-well plates (2 wells/transfection) for β-galactosidase assays. The next morning, cells used for cAMP assays were labelled for 4-6 hours with 2-3μCi/well [^{3}H]-adenine (ICN, Costa Mesa, CA). Just prior to the cAMP assay, companion cells for β-galactosidase assays were lysed in 500μl of buffer B (100mM KH_2PO_4 pH 7.8, 0.2% Triton X-100, 1mM DTT) and frozen until use. cAMP and β-galactosidase assays were carried out as described and all raw data was normalized to the measured β-galactosidase signal for each transfection.

β-Galactosidase Assay- Lysates of transiently transfected cells (described above) were thawed and centrifuged at 16,000x*g* for 10 minutes. The supernatant (20μl) was

combined with 100µl of reaction buffer (100mM Na_2HPO_4 pH 8.0, 1mM $MgCl_2$, 35mM Galacton (Tropix, Bedford, MA), 100mM D-galactose) and incubated in the dark at room temperature for 60 minutes. During this incubation period, a 10% solution of Emerald (Tropix, Bedford, MA) in 0.2N NaOH was prepared for subsequent addition to the samples at 5 second intervals by a Berthold luminometer. Each well of lysed cells was assayed in duplicate and data was used to normalize for transfection efficiency.

Preparation of membranes from mouse tissues and HEK 293 cells- C57BL/6J mice were sacrificed by decapitation and tissues were dissected and frozen on dry ice as rapidly as possible. Isolated tissues were frozen at -80°C until use. For membrane preparation, tissues were thawed on ice, placed in 5-10 volumes of ice-cold buffer A (20mM Tris-HCl pH 7.4, 1mM EDTA, 2mM $MgCl_2$, 0.5mM DTT, 0.5mM PMSF, 3.2µg/ml leupeptin, 2µg/ml aprotinin, 0.5µg/ml pepstatin A), and disrupted with a hand-held polytron for 15-30 seconds. Disrupted tissue was then subjected to Dounce homogenization (30 strokes). Homogenates were centrifuged at 600xg for 5 minutes and the supernatant was retained. This supernatant was centrifuged at 90,000xg for 30 minutes. Membrane pellets were resuspended in buffer B (50mM sodium phosphate pH 7.4, 10mM EDTA, 1mM DTT, 2mM $MgCl_2$, 0.5mM PMSF, 5µg/ml leupeptin, 2µg/ml aprotinin) and either used immediately or frozen at -80°C until use. For HEK 293 cell membranes, cells were grown to confluence in 100mm culture dishes and harvested in buffer A. Centrifugation steps were carried out as described for the mouse tissues. Membrane pellets were resuspended in buffer B and either used immediately or frozen at -80°C until use. Protein concentration was determined according to the method of Bradford (Bradford, 1976) using BSA as the standard.

Western analysis of AC4 expression- 50μg of mouse tissue membranes or 20μg of HEK 293 cell extracts were subjected to deglycosylation prior to electrophoresis. Briefly, for membranes from tissues or HEK 293 cells treated with N-glycosidase F (Boehringer Mannheim, Indianapolis, IN), samples were resuspended in buffer B and SDS was added to 0.1%. Following SDS addition, sample were denatured by heating to 95°C for 5 minutes. After denaturation, Nonidet P-40 was added to a final concentration of 0.8%. Samples were deglycosylated with 0.5 units of N-glycosidase F for 1 hour at 37°C. The deglycosylation reaction was terminated by addition of Laemmli buffer. Proteins were separated by SDS-PAGE through 7.5% acrylamide gels. Proteins treated with or without N-glycosidase F were transferred to nitrocellulose and blocked with 3% cold fish gelatin (Sigma, St. Louis, MO) in 20mM Tris-HCL pH 7.4/150mM NaCl/0.05% Tween 20 (TBST) for 1-2 hours at room temperature. Following block, blots were incubated with 100ng/ml anti-AC4 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 16-20 hours. Blots were developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer's guidelines.

Immunohistochemistry- C57Bl/6J mice were killed by decapitation and brains were quickly removed. Brain slices (500μm) were cut in the coronal plane (or sometimes the transverse plane for hippocampal slices) using a vibratome (Campden Instruments Ltd., U.K.). The slices were fixed for 4-6 hours in 4% paraformaldehyde/PBS pH 7.4. Following fixation, slices were cryoprotected in PBS/30% sucrose and maintained at 4°C until further use. To prepare brain sections for immunostaining, 40μM sections were cut on a sliding microtome from the fixed 500μM slices and placed, free-floating, directly into a blocking solution of PBS pH 7.4/0.025% Triton X-100 (PBST) supplemented with 5% BSA. Slices were blocked for 1-2 hours at room temperature and then incubated free-floating in PBST/0.5% BSA containing 100ng/ml rabbit polyclonal antibodies against AC2 or AC4 (Santa Cruz

Biotechnology, Santa Cruz, CA) for 18-24 hours. When indicated, peptide antigens were at a 20-fold molar excess to antibody and were preadsorbed to the antibody for 8-12 hours at 4°C. Following primary antibody, sections were washed five times for 10 minutes in PBST at room temperature, after which a 1:500 dilution of goat anti-rabbit IgG conjugated to Texas Red (Jackson Laboratories, West Grove, PA) was added to the sections for 1-2 hours at room temperature. Sections were then washed as described above for the primary antibody and mounted onto slides. Immunostaining was analyzed using a Bio-Rad MRC 600 confocal microscope (William M. Keck Imaging Center, University of Washington). Labeling of discrete brain structures was determined according to the mouse brain atlas of Franklin and Paxinos (Academic Press, San Diego). Images were processed on a Macintosh Power Mac using Adobe Photoshop 4.0.

Results

G protein-coupled receptors stimulate AC4 in vivo- To determine if AC4 is activated in intact cells by G_S-coupled receptors, HEK 293 cells stably expressing AC4 were treated with the β-adrenergic agonist isoproterenol. 1μM isoproterenol stimulated cAMP levels by 3.5-fold in AC4-expressing cells (Figure 23A). The basal cAMP levels in AC4-expressing cells was elevated ~4-fold relative to vector transfected cells. To determine if AC4 is stimulated by other G_S-coupled receptors, we also measured cAMP responses to serotonin in cells stably expressing the G_S-coupled 5HT7 receptor and AC4. In 5HT7/AC4 cells, serotonin stimulated cAMP levels, with maximal stimulation occurring at 250nM serotonin (Figure 23B).

In vitro, βγ subunits potentiate $G_{S\alpha}$ stimulation of AC2 (Tang and G., 1991; Taussig et al., 1993) and AC4 (Gao and Gilman, 1991). In addition, βγ stimulation has also been demonstrated for AC2 *in vivo* (Federman et al., 1992; Koch et al., 1994; Lustig

et al., 1993). To determine if βγ from G_i stimulates AC4 in intact cells, we first treated HEK 293 cells expressing AC4 with pertussis toxin. Pertussis toxin catalyzes the ADP-ribosylation of the G_i/G_o class of G proteins (Burns et al., 1983; Hsia et al., 1984; Katada and Ui, 1982; Katada and Ui, 1982; Neer et al., 1984) leading to uncoupling of the receptor and G_i (West et al., 1985). In cells not treated with pertussis toxin, isoproterenol stimulated AC4 3-fold. Activation of the G_i-coupled somatostatin receptor did not stimulate AC4. However, coapplication of isoproterenol and somatostatin produced a synergistic increase in cAMP levels over those of isoproterenol alone (Figure 24A). In pertussis toxin-treated AC4-expressing cells, isoproterenol stimulated cAMP levels 2-fold. The somatostatin potentiation of isoproterenol-stimulated cAMP levels was completely blocked by pertussis toxin pretreatment (Figure 24A).

To further implicate βγ release from G_i-coupled somatostatin receptors in potentiation of G_s-stimulated AC4, we carried out transient transfections of HEK 293 cells in which the G_s-coupled 5HT7 receptor (Shen et al., 1993) and AC4 were cotransfected with empty vector or a construct encoding the βγ-binding, carboxy-terminal region of β-adrenergic receptor kinase 1 (βARK1-ct). Cellular expression of βARK1-ct has been shown to attenuate βγ effects on the MAP kinase pathway and phospholipase C (Inglese et al., 1994; Koch et al., 1994; Luttrell et al., 1995). In cells transfected with 5HT7 and AC4 plus empty vector, somatostatin potentiated serotonin stimulation of AC4 by ~80% (Figure 24B). When βARK1-ct was coexpressed, somatostatin potentiation of G_s-stimulated AC4 was completely abolished (Figure 24B). Similar results were also obtained using transducin-α as the βγ scavenger (see Chapter 2). This demonstrated that βγ release from G_i can potentiate activation of G_s-stimulated AC4 *in vivo*.

Characterization of antibodies to AC2 and AC4- To characterize polyclonal antibodies to AC2 or AC4, we determined the specificity of this antibody for immunohistochemical detection of AC2 or AC4. Using plain HEK 293 cells or HEK 293 cells expressing either AC2 or AC4, we found that the AC2 antibody only stained cells expressing AC2 (Figure 25B), but not vector-transfected cells (Figure 25A). Similarly, the AC4 antibody stained cells expressing AC4 (Figure 26B), but not vector-transfected cells (Figure 26A). In addition, preadsorbtion of the peptide antigens to the antibodies for AC2 and AC4 blocked the staining (Figures 25C and 26C, respectively).

Figure 27 shows a representative western blot in which untransfected cells show no immunoreactive bands at the expected molecular weight for adenylyl cyclases, while cells expressing AC4 reveal bands at 110 kD and 150 kD. The lower band is non-glycosylated AC4 since treatment with N-glycosidase F shifted protein in the 150 kD band to the lower molecular weight band size. This result , coupled with the immunohistochemistry data from HEK 293 cells (Figure 26) demonstrate that this antibody is specific for AC4. Furthermore, this antibody does not recognize other adenylyl cyclases expressed in HEK 293 cells (L. Prichard, L.P. Baker, M.D. Nielsen, and D.R. Storm, unpublished observations). However, upon characterizing the AC2 antibody, we found that this antibody was unable to recognize recombinant AC2 by Western analysis (data not shown). This could be due to the fact that the antibody requires a native conformation for epitope recognition, and SDS denaturation prevents the AC2 antibody from binding to AC2 protein.

Antibodies to AC4 recognize AC4 protein in mouse lung- In addition to characterizing the AC4 antibody in HEK 293 cells, it was of interest to determine if AC4 antibodies were capable of recognizing native AC4, especially in tissues where AC4 mRNA has been reported to exist such as brain and lung (Gao and Gilman, 1991; Pian and Dobbs, 1995). Membranes prepared from a variety of mouse tissues were treated with or without N-

glycosidase F and subjected to SDS-PAGE through 7.5% acrylamide gels and subsequent western blotting. Blots revealed the presence of AC4 protein in lung (Figure 27), while heart, liver, kidney, and spleen were negative (data not shown). In lung, AC4 antibodies recognized a diffuse band at 140kD, which is close to the molecular weight of the mature protein expressed in HEK 293 cells. This band was a glycosylated protein since its apparent molecular weight shifted down to ~105kD when treated with N-glycosidase F. Unexpectedly, we were not able to repeat an earlier result in which a diffuse band at 160 kD was recognized in brain. In any case, the detection of AC4 protein in lung is the first demonstration of the existence of AC4 protein in native tissue.

Immunohistochemical analysis of AC2 and AC4 in mouse hippocampus-

To determine the localization of AC2 and AC4 proteins in mouse hippocampus, 40μM brain slices containing the hippocampus were processed for immunohistochemistry. Staining of slices with the AC2 antibody revealed moderate to high levels of immunoreactivity in dentate gyrus (Figure 28A) and CA1 (Figure 28B), and weak staining in CA3 (data not shown). Immunostaining was blocked by preadsorbtion of the peptide antigen to the antibody prior to staining (Figures 28C-D). On closer observation, AC2 staining along basal dendrites in the stratum radiatum was punctate, possibly existing at sites of synaptic contact. Cell bodies in CA1 were also stained. In dentate gyrus, staining intensity was equivalent between cell bodies and dendrites.

The staining pattern observed for AC4 in hippocampus was somewhat different than that for AC4. Moderate to high levels of staining were observed in dentate gyrus, CA3, and CA1 (Figure 29A-C). In dentate gyrus and CA3, cell bodies and their associated processes stained positively. Scattered cells in the hilus were also stained (Figure 29A). In CA1, staining was prominent in stratum radiatum dendrites (Figure 29B), although some cell bodies showed positive immunoreactivity as well. Pyramidal cells and their dendrites

in the transition zone between CA1 and subiculum were also strongly stained (Figure 29F). AC4 immunoreactivity was prevented by preadsorbtion of the peptide antigen to the antibody (Figures 29D and 29E). Taken together, these results with AC2 and AC4 immunostaining in mouse hippocampus demonstrate that βγ-stimulated adenylyl cyclases are expressed throughout the hippocampus.

Immunohistochemical analysis of AC4 in other brain regions- To examine the expression patterns of AC4 in other areas of brain, coronal sections of mouse forebrain were processed for immunocytochemistry. An extensive analysis of AC2 mRNA distribution in brain has been previously reported (Furuyama et al., 1993). Immunostaining with the AC4 antibody was quite enlightening since there is no information concerning the distribution of AC4 in brain. In neocortex, a moderate percentage of neurons were positively labeled, including pyramidal cell bodies and their dendrites (Figures 30A and 30B). Moderate immunoreactivity was also observed in the piriform cortex (Figure 30C). Discrete labeling of neurons in the septum (Figure 30D), medial habenular nucleus (Figure 30E), induseum griseum (Figure 30F), paraventricular thalamic nucleus (Figure 30G), and a small nucleus just lateral to the intrabulbar portion of the anterior commissure (possibly part of the dorsal endopiriform nucleus) (Figure 30H) were also observed. Although there is no obvious correlation between the AC4 staining pattern and a functional parameter (i.e., similar to AC1 distribution in areas of the brain associated with learning and memory), it is intriguing to note that several areas stained with the AC4 antibody are sites of synaptic plasticity (e.g., neocortex, piriform cortex) (Cruikshank and Weinberger, 1996; Hasselmo and Barkai, 1995).

Discussion

Adenylyl cyclase is stimulated by hormones coupled to G_S and inhibited by hormones coupled to G_i. In brain and lung, however, G_i-coupled receptors can potentiate rather than inhibit G_S-stimulated cAMP levels (Andrade, 1993; Gereau IV and Conn, 1994; Pian and Dobbs, 1995). In hippocampus, in particular, several groups have observed potentiation of G_S-coupled receptor stimulation by G_i-coupled receptors (Andrade, 1993; Gereau IV and Conn, 1994). The hippocampus is an important loci for processes underlying learning and memory and the cAMP signal transduction pathway plays an important role in synaptic plasticity (Frey et al., 1993; Huang et al., 1995; Huang et al., 1994; Impey, 1996; Wu et al., 1995). Recent evidence suggests that the Ca^{2+}-stimulated adenylyl cyclases AC1 and AC8 may be involved in synaptic plasticity (Wu et al., 1995: Xia et al., 1995) however, detection of mRNA in hippocampus for at least one $\beta\gamma$-stimulated adenylyl cyclase, AC2 (Furuyama et al., 1993), suggests that there may be additional mechanisms for generating robust cAMP increases thought to be important for mechanisms underlying synaptic plasticity (Backsai et al., 1993; Hagiwara et al., 1993). The goals of this research were to characterize the regulatory properties of the $\beta\gamma$-stimulated AC4 *in vivo*, as well as determining the immunohistochemical localization of AC2 and AC4 in brain.

In this study, we demonstrate that AC4 is stimulated by G_S-coupled receptors in intact cells. This is consistent with previous *in vitro* data which demonstrated that AC4 was stimulated by recombinant $G_{S\alpha}$ (Gao and Gilman, 1991). Additionally, we found that G_i-coupled receptor activation potentiates the response of AC4 to G_S-coupled receptors in vivo. Specifically, stimulation of AC4 by G_S-coupled receptors was potentiated by activation of G_i-coupled somatostatin receptors. Somatostatin potentiation of isoproterenol-stimulated AC4 was prevented by pertussis toxin, while in transient

transfection experiments, coexpression of the COOH-terminal, βγ-binding region of βARK_1 effectively blocked somatostatin potentiation of 5HT7-stimulated AC4. This demonstrates that AC4 responds in vivo to paired G_s and G_i inputs with βγ suprastimulation. Similar findings have been previously observed with AC2 (Federman et al., 1992; Koch et al., 1994; Lustig et al., 1993). These results demonstrate that both AC2 and AC4 can function as coincidence detectors of paired G_s and G_i inputs. Additionally, AC2 can detect simultaneous activation of protein kinase C and G_i (Tsu and Wong, 1996). Since coincidence detection of separate, temporally overlapping signals is thought to be important for neuroplasticity (Bourne and Nicoll, 1993), the results here suggest a role for both AC2 and AC4 in synaptic plasticity.

A second aspect of this study was directed at elucidating the immunohistochemical localization of AC2 and AC4 in mouse hippocampus and other brain regions. To this end, we have found that both AC2 and AC4 are expressed in hippocampus. In hippocampus, AC2 was most abundantly expressed in dentate gyrus and CA1, with weaker labeling in CA3. In CA1, AC2 staining occurred in a punctate manner along basal dendrites in the stratum radiatum. From these data, we cannot discern whether the punctate AC2 staining in stratum radiatum is localized pre- or post-synaptically. Future studies employing double-labeling techniques using axonal and dendritic marker will be required to resolve this question.

Regarding AC4, immunohistochemical labeling in hippocampus was identified in dentate gyrus, CA3, and CA1. In particular, in CA1, staining was predominant in basal dendrites in the stratum radiatum, although cell bodies were also labeled albeit to a lesser degree. These results demonstrate the presence of AC2 and AC4 in hippocampus, especially in stratum radiatum in CA1, and suggest a role for the βγ-stimulated adenylyl cyclases in synaptic plasticity.

During this study, we also determined the immunohistochemical localization of AC4 in other areas of mouse forebrain. Our data concerning distribution of AC4 shows that AC4 is not widely expressed in mouse forebrain, but expressed mainly in discrete cell groups. Except for a general, low to moderate level of staining in neocortex, brain regions exhibiting AC4 immunoreactivity included piriform cortex, medial septal region, medial habenular nucleus, paraventricular thalamic nucleus, and induseum griseum. Interestingly, several brain regions expressing AC4 including neocortex and piriform cortex also exhibit various forms of synaptic plasticity. Therefore, involvement of AC4 in mechanisms underlying neuroplasticity in these brain areas seems possible.

What might be the role of βγ-stimulated adenylyl cyclases in synaptic plasticity? AC2 may serve to integrate inputs from multiple signalling pathways, since it can respond to either $G_{s\alpha}$ or PKC and βγ from G_i with suprastimulation *in vivo* (Federman et al., 1992; Lustig et al., 1993; Tsu and Wong, 1996). Additionally, PKC may suppress the responsiveness of AC2 to paired $G_{s\alpha}$ and βγ stimulation (Zimmermann and Taussig, 1996). These regulatory features impinging upon AC2 may generate a cAMP signal with the necessary spatial and temporal characteristics to contribute to the overall robust cAMP signal thought to be necessary for mechanisms underlying long-term changes in synaptic strength (Backsai et al., 1993; Hagiwara et al., 1993) (See Figure 31). Consistent with this notion, mf-CA3 LTP appears to be dependent on activation of G_i/G_o-coupled opioid receptors (Williams and Johnston, 1996) and pertussis toxin sensitive G proteins (Ito et al., 1988) Therefore, pertussis toxin sensitive G proteins may be an important component of long-lasting enhancement of synaptic transmission at the mf-CA3 synapse. Furthermore, since PKC likely plays a role in long-term potentiation (Abeliovich et al., 1993; Malinow et al., 1989; Wang and Feng, 1992), perhaps its action on AC2 is an important component of PKC's involvement in neuroplasticity.

The possible role of AC4 in synaptic plasticity in the hippocampus may be to simply integrate G_S and G_i inputs, perhaps as a complement to the signal generated by AC2 (see Figure 31). Although it is difficult to discern from our data, it is possible that AC2 and AC4 are localized to different regions along CA1 dendrites. A differing localization of AC2 and AC4 may allow for independent generation of cAMP signals along a dendrite, possibly to segregate the effects of PKC and G_S on cAMP levels. At any rate, the presence of AC2 and AC4 in the hippocampus allows for flexibility in the generation of robust cAMP increases necessary for synaptic plasticity. Instead of relying on the Ca^{2+}-stimulated adenylyl cyclases as the sole means of eliciting cAMP-mediated long-term adaptive changes in neurons, hippocampal neurons may have evolved with several distinct mechanisms for expression of synaptic plasticity. Perhaps there are two parallel pathways involving cAMP which encode different forms of synaptic plasticity.

In summary, these results demonstrate that AC4 can act as a coincidence detector of paired G_S and G_i inputs. The finding that both AC2 and AC4 proteins are expressed in hippocampus, and in particular, in CA1 dendrites, suggests that these adenylyl cyclases may play a role in certain forms of hippocampal synaptic plasticity. To obtain a clearer understanding of the physiological roles of AC2 and AC4, gene disruption experiments may be required.

Figure 23. G_S-coupled receptors stimulate AC4 in HEK 293 cells. A, HEK 293 cells stably expressing AC4 were treated with vehicle or 1μM isoproterenol. B, HEK 293 cells stably coexpressing AC4 with the G_S-coupled 5HT7 receptor were treated with the indicated concentrations of serotonin. Relative cAMP accumulation was determined as described under "Materials and Methods" and endogenous HEK 293 cell cAMP accumulation was subtracted from the cAMP measured in AC4 expressing cells. The data are the means +/- S.D. of triplicate assays.

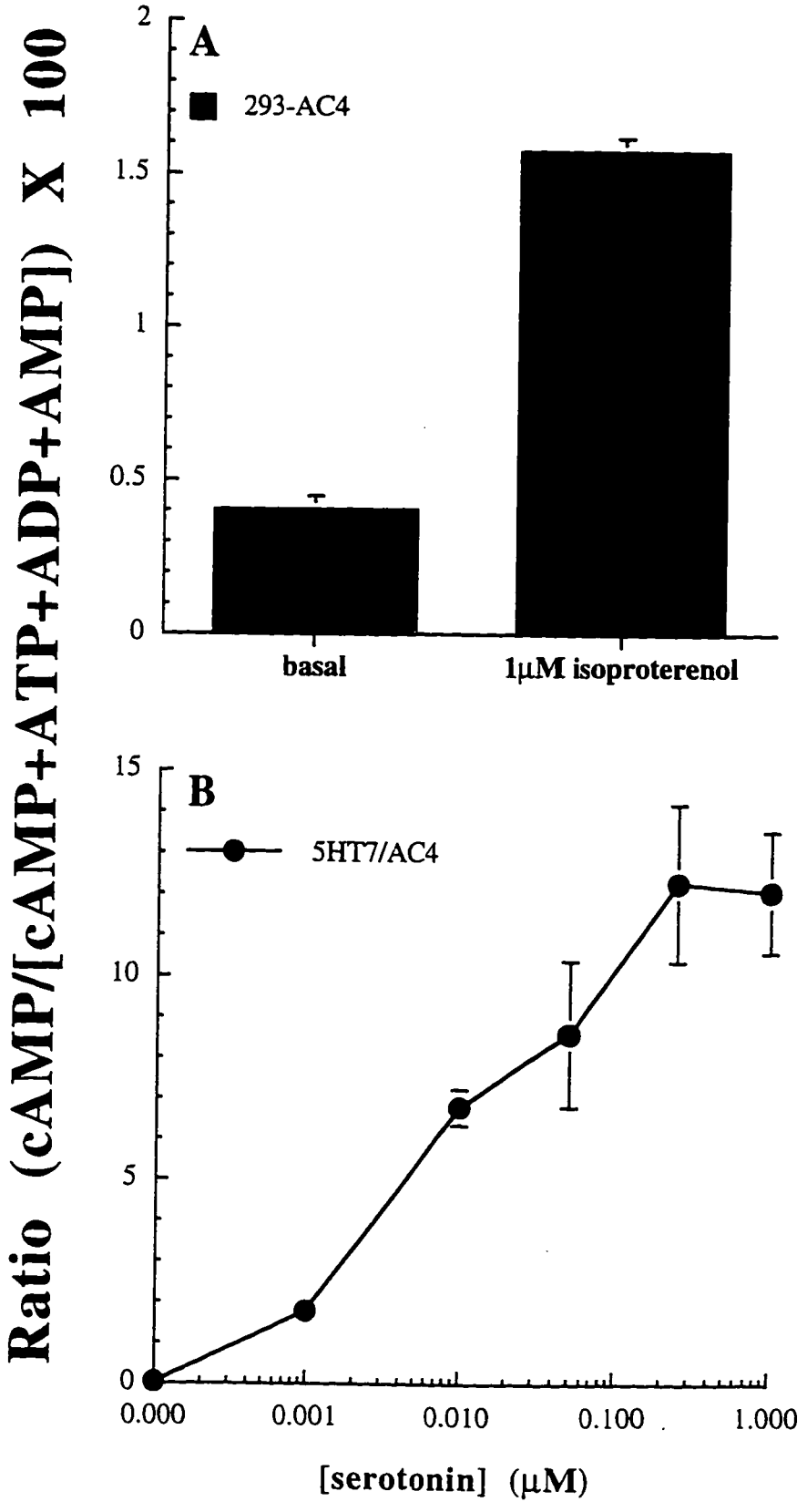
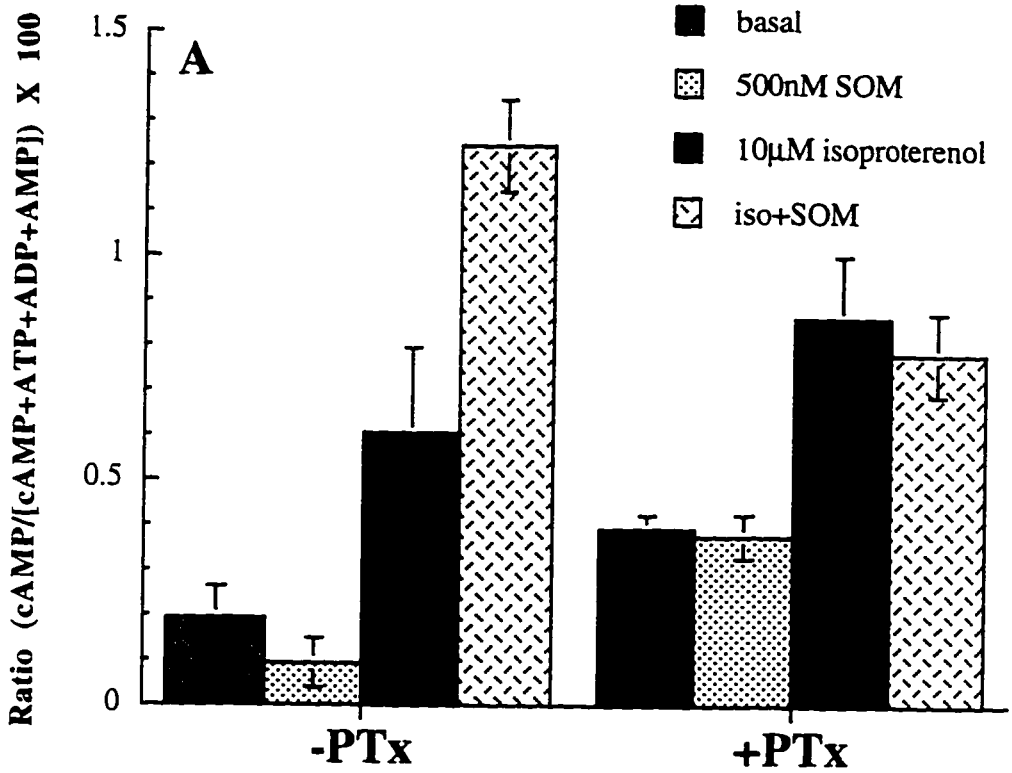

Ratio ([cAMP]/[cAMP+ATP+ADP+AMP]) X 100
A
293-AC4
2
1.5
1
0.5
0
basal
1μM isoproterenol
B
5HT7/AC4
15
10
5
0
0.000
0.001
0.010
0.100
1.000
[serotonin] (μM)

Figure 24. Pertussis toxin and βARK$_1$-ct minigene expression block G_i-coupled receptor potentiation of G_s-stimulated AC4. A, HEK 293 cells stably expressing AC4 were incubated overnight with vehicle (-PTx) or 200ng/ml pertussis toxin (+PTx). The following day cells were treated with vehicle or 10μM isoproterenol in the presence or absence of 500 nM somatostatin (SOM). Relative cAMP accumulation was determined as described under "Materials and Methods." The data are the means +/- S.D. of triplicate assays. B, HEK 293 cells were transiently transfected with both RSV-β-galactosidase and the 5HT7 receptor and either pCDNAIII or βARK$_1$-ct, as well as pCEP4 or pCEP4-AC4 as described under "Materials and Methods." Cells transfected with βARK$_1$-ct are denoted as "PH domain." AC4-transfected cells with or without βARK$_1$-ct were treated with 10μM serotonin in the presence or absence of 500nM somatostatin (SOM). Relative cAMP accumulation was determined as described under "Materials and Methods." Data are expressed as percent cAMP accumulation in the absence of somatostatin, with this level being set as 100%. The -fold stimulation over the basal activities was similar between transfections with or without βARK$_1$-ct. The data are the means +/- S.D. of triplicate assays and were subtracted for endogenous (pCEP4 transfectants) cAMP accumulation and corrected for transfection efficiency using β-galactosidase.


A
basal
500nM SOM
10μM isoproterenol
iso+SOM
Ratio (cAMP/[cAMP+ATP+ADP+AMP]) X 100
1.5
1
0.5
0
-PTx
+PTx

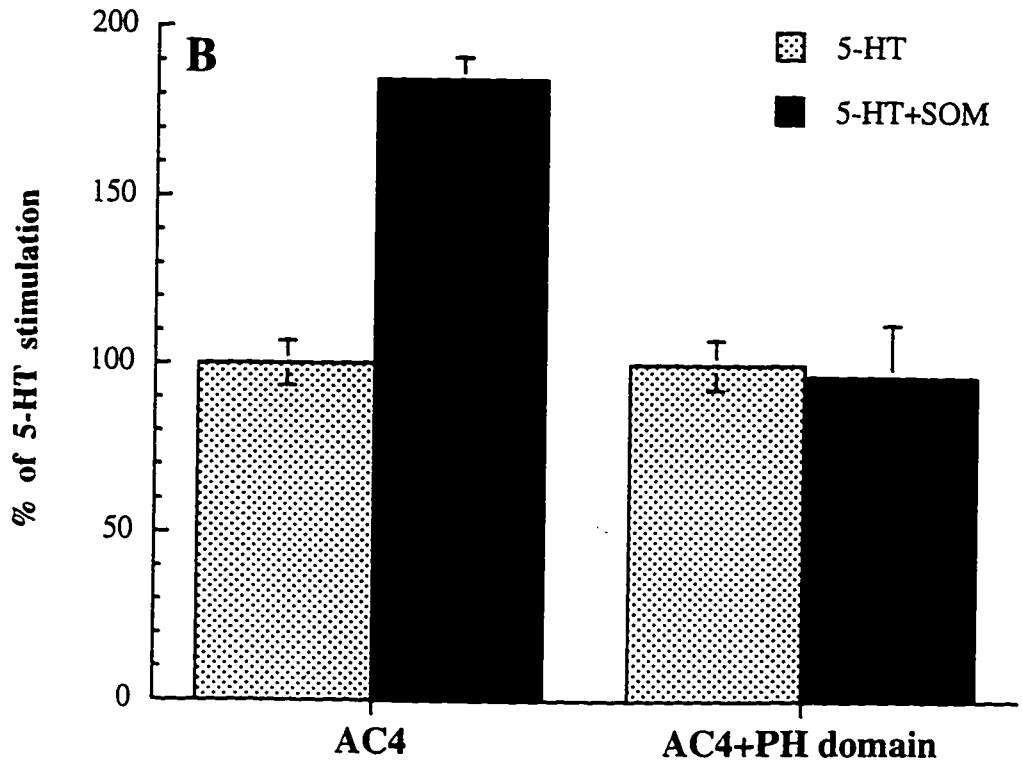

B
5-HT
5-HT+SOM
% of 5-HT stimulation
200
150
100
50
0
AC4
AC4+PH domain

Figure 25. Immunohistochemical detection of AC2 stably expressed in HEK 293 cells. A, HEK 293 cells tranfected with the pCEP4 vector (designated HEK 293) were processed for immunohistochemistry with the AC2 antibody as described under "Materials and Methods". B, HEK 293 cells expressing AC2 (designated 293-AC2) were stained with the AC2 antibody. C, Preadsorbtion of the AC2 peptide antigen AC2 antibody blocked AC2 immunostaining in HEK 293 cells expressing AC2.

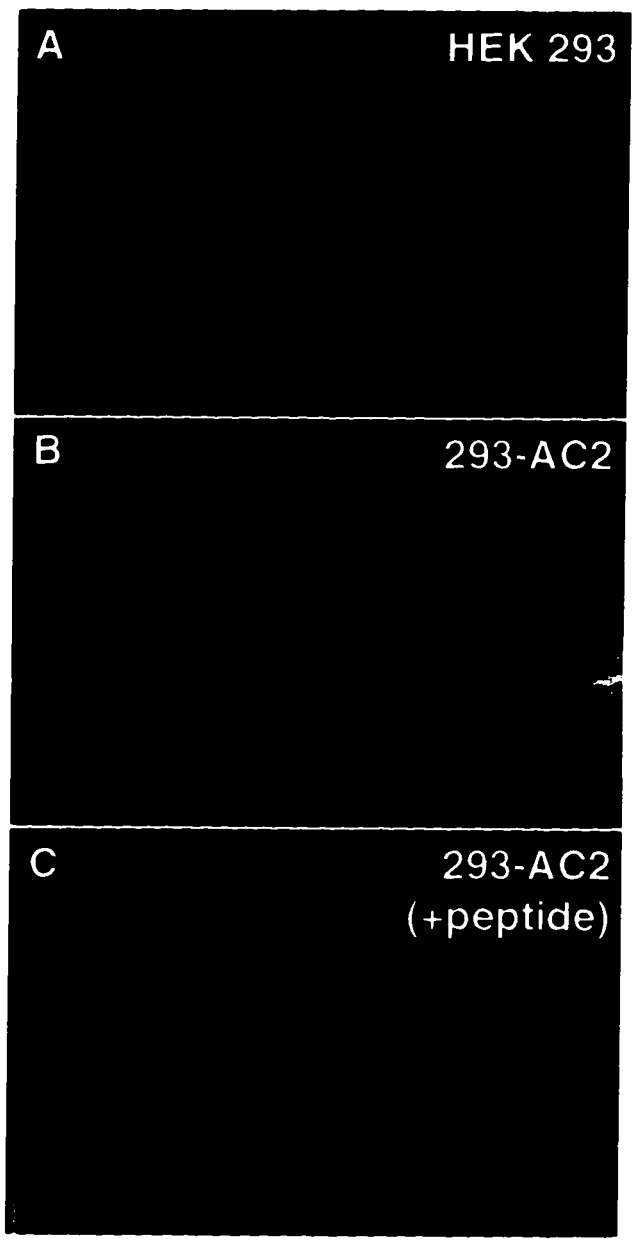

A
HEK 293
B
293-AC2
C
293-AC2
(+peptide)

Figure 26. Immunohistochemical detection of AC4 stably expressed in HEK 293 cells. A, HEK 293 cells tranfected with the pCEP4 vector (designated HEK 293) were processed for immunohistochemistry with the AC4 antibody as described under "Materials and Methods". B, HEK 293 cells expressing AC4 (designated 293-AC4) were stained with the AC4 antibody. C, Preadsorbtion of the AC4 peptide antigen AC4 antibody blocked AC4 immunostaining in HEK 293 cells expressing AC4.

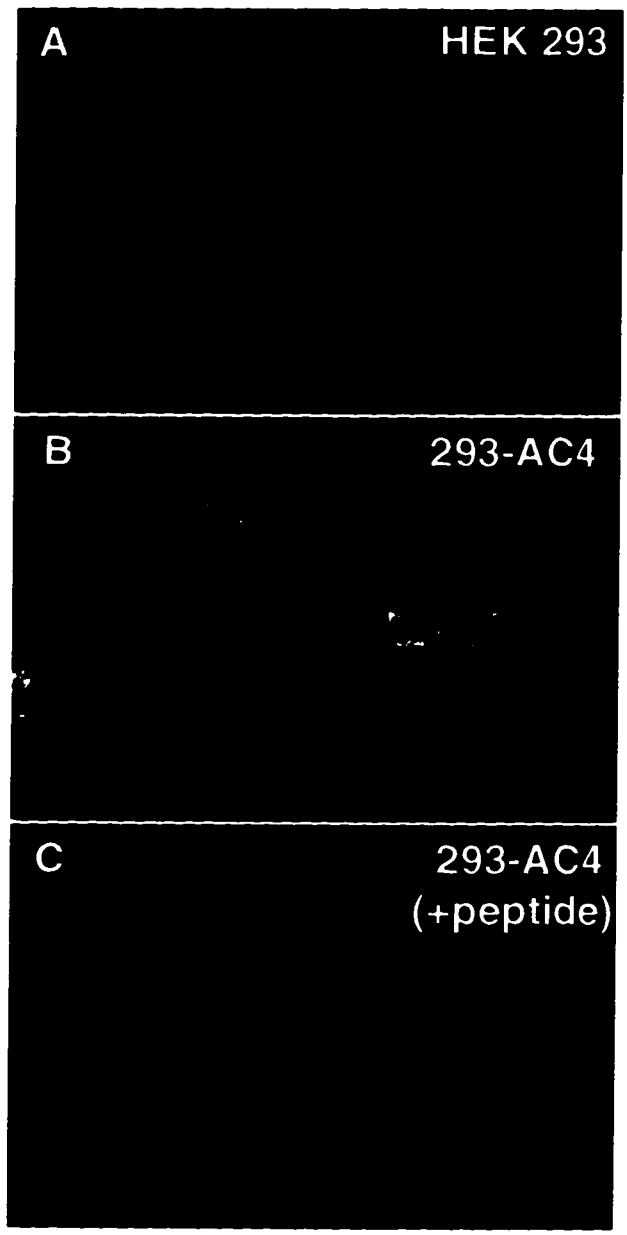

A
HEK 293
B
293-AC4
C
293-AC4
(+peptide)

Figure 27. Western blot detection of AC4 in membranes from HEK 293 cells and mouse lung. Membranes from several mouse tissues were prepared and treated with or without N-glycosidase F as described under "Materials and Methods." 20μg of membranes from 293-pCEP4 cells (lanes 1 and 2), 293-AC4 cells (lanes 3 and 4), or 50μg of membrane protein from lung (lanes 5 and 6) were separated by SDS-PAGE through 7.5% acrylamide gels. Protein was transferred to nitrocellulose and incubated with anti-AC4 antibodies. Blots were developed using enhanced chemiluminescence and scanned on a Hewlett Packard Scan Jet II CX scanner.

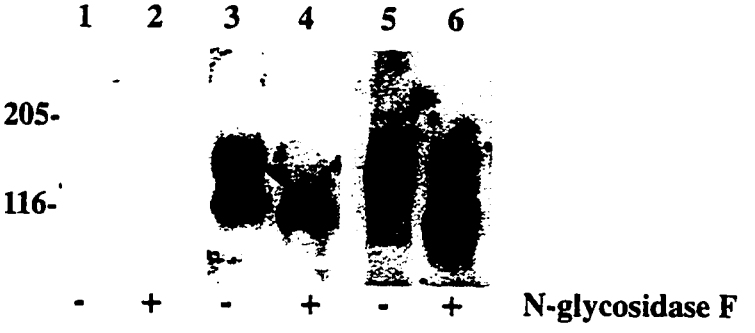

1 2 3 4 5 6
205-
116-
- + - + - +
N-glycosidase F

Figure 28. Immunohistochemical detection of AC2 in mouse hippocampus. Mouse brain sections (40μm) were processed for immunohistochemistry as described under "Materials and Methods." A, AC2 immunostaining in dentate gyrus (designated DG). Magnification, 600X. B, AC2 immunostaining in area CA1. Magnification, 200X. Note that the stratum radiatum layer is designated Str. Rad. C, Block of AC2 labeling in dentate gyrus by AC2 peptide antigen. Magnification, 200X. D, Block of AC2 labeling in area CA1 by AC2 peptide antigen. Magnification, 200X.

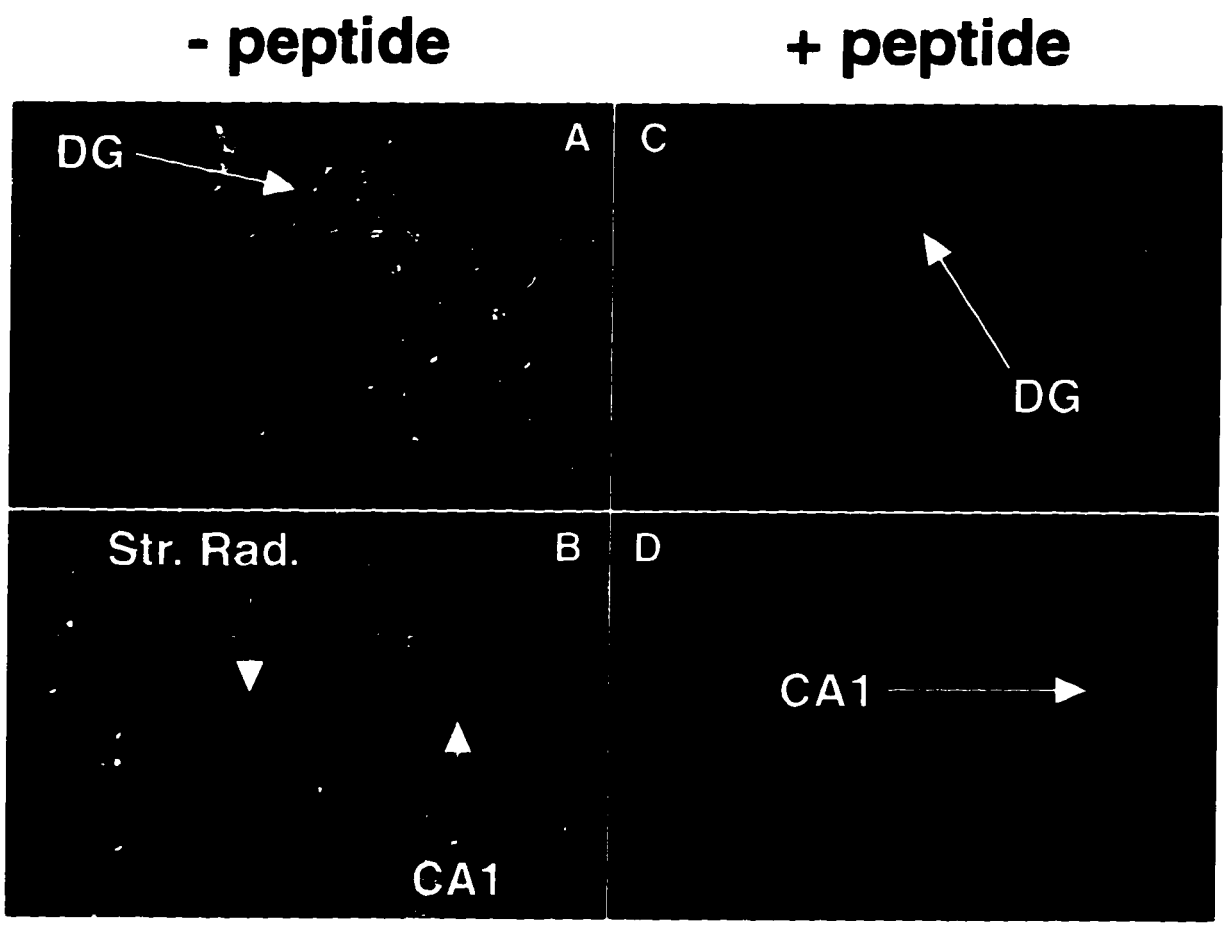

- peptide
+ peptide
DG
A
C
DG
Str. Rad.
B
D
CA1
CA1

Figure 29. Immunohistochemical detection of AC4 in mouse hippocampus. Mouse brain sections (40μm) were processed for immunohistochemistry as described under "Materials and Methods." A, AC4 immunostaining in dentate gyrus (designated DG). The hilus is designated as "Hil". Magnification, 200X. B, AC4 immunostaining in area CA1. Magnification, 200X. Note that the stratum radiatum layer is designated Str. Rad.). C, AC4 immunostaining in area CA3. Magnification, 200X. D, Block of AC4 immunostaining in dentate gyrus by AC4 peptide antigen. E, Block of AC4 immunostaining in area CA1 by AC4 peptide antigen. Magnification, 200X. F, AC4 immunostaining in the CA1/subiculum transition area (designated CA1/sub.). Magnification, 200X.

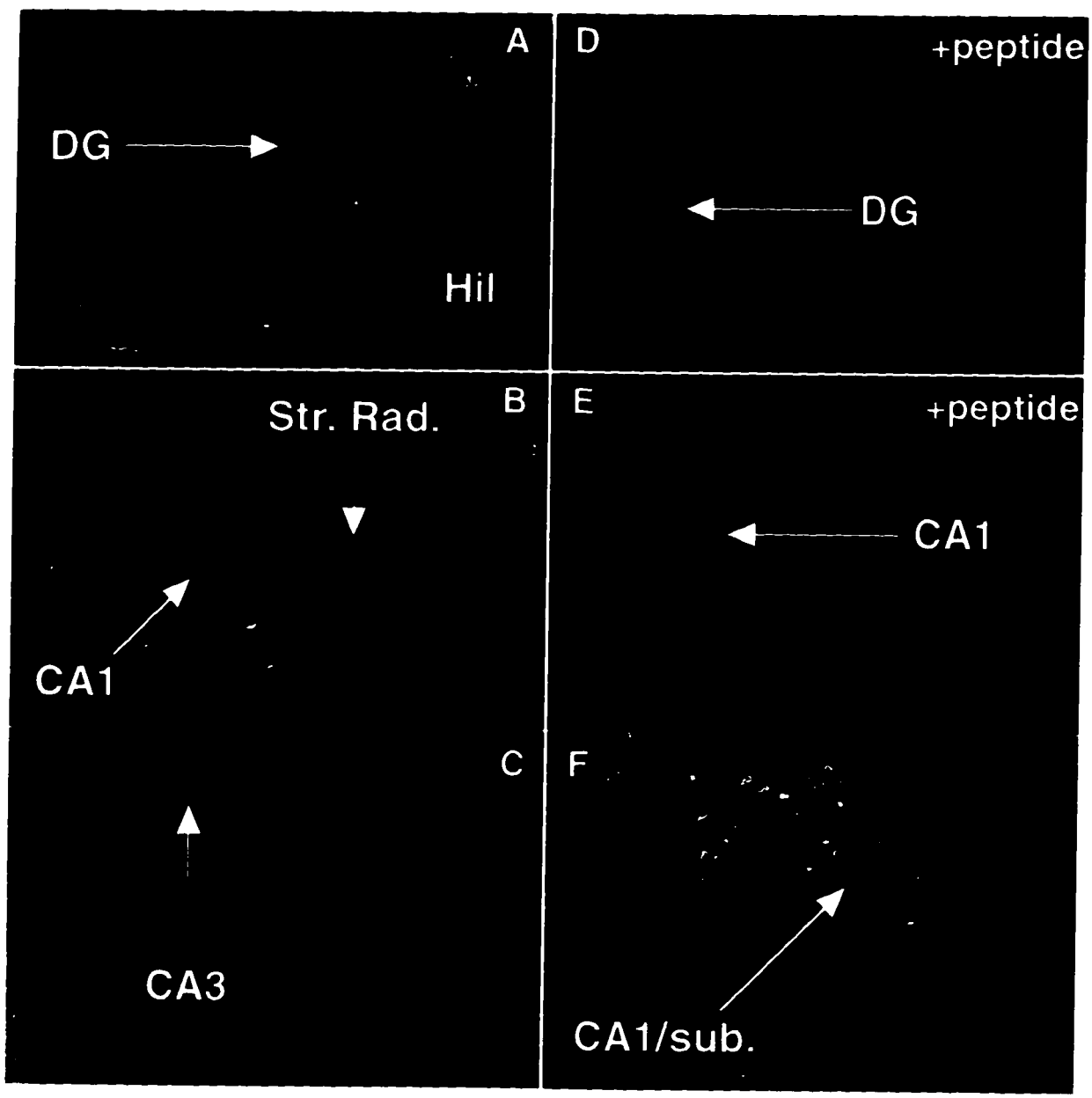

A
DG
Hil
D
+peptide
DG
Str. Rad.
B
CA1
C
CA3
E
+peptide
CA1
F
CA1/sub.

Figure 30. Immunohistochemical detection of AC4 in discrete areas of mouse brain. Mouse brain sections (40μm) were processed for immunohistochemistry as described under "Materials and Methods." A, AC4 immunostaining in neocortex. Magnification 200X. B, AC4 immunostaining in neocortical dendrites. Magnification 600X. C, AC4 immunostaining in the piriform cortex. Magnification, 200X. D, AC4 immunostaining in the septum. Magnification, 200X. E, AC4 immunostaining in the medial habenular nucleus. Magnification, 300X. F, AC4 immunostaining in the induseum griseum. Magnification, 160X. G, AC4 immunostaining in the paraventricular thalamic nucleus. Magnification, 200X. H, AC4 immunostaining in cells located between the intrabulbar portion of the anterior commissure and the dorsal endopiriform nucleus. Magnification, 600X.

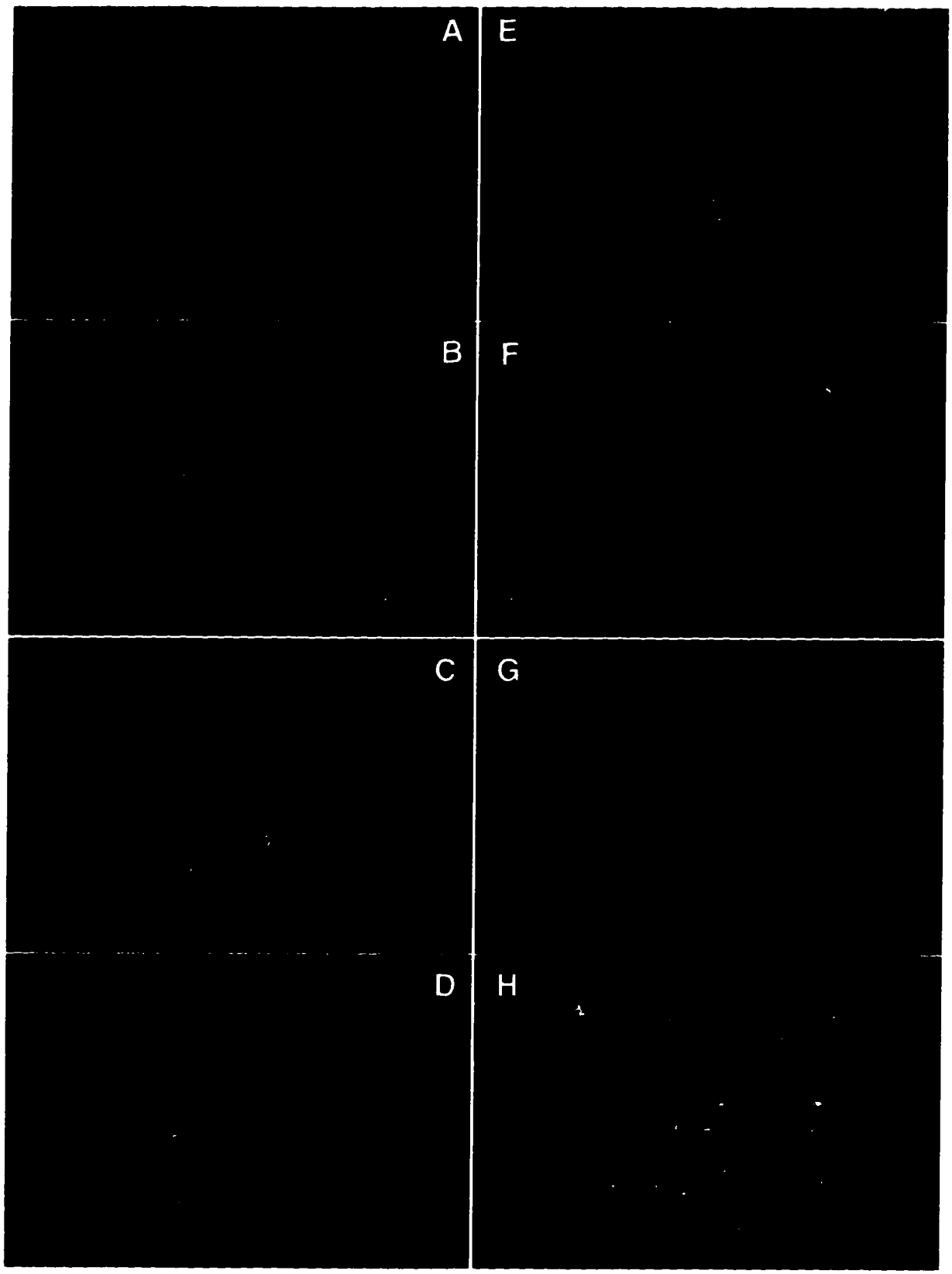
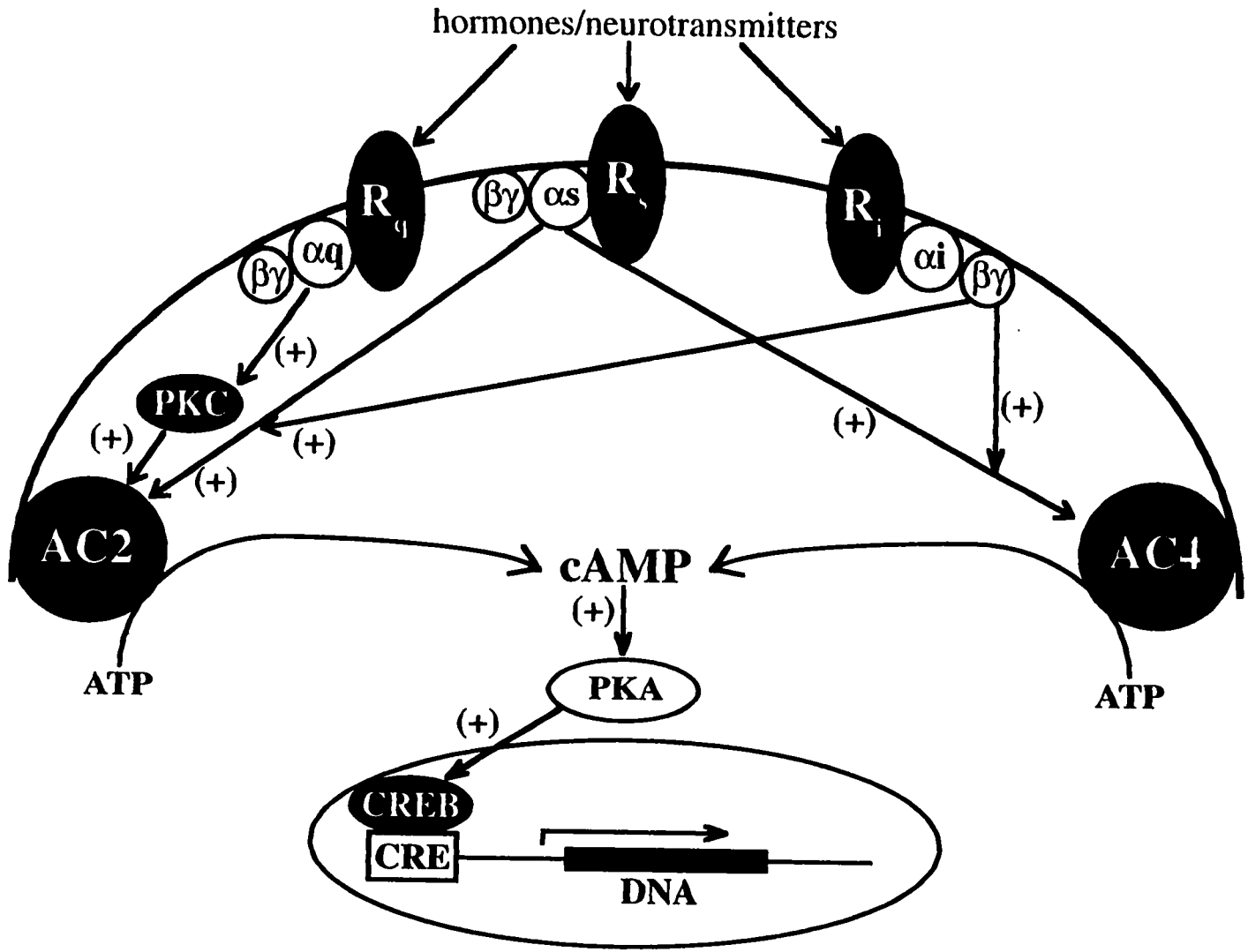

A
E
B
F
C
G
D
H

Figure 31. Proposed involvement of βγ-stimulated adenylyl cyclases AC2 and AC4 in mechanisms of synaptic plasticity in the hippocampus. AC2 can integrate signals from $G_{s\alpha}$, $G_{i\beta\gamma}$, and PKC and produce robust increases in cAMP which may then stimulate cAMP-responsive transcription. AC4 can detect coincident signals from $G_{s\alpha}$ and $G_{i\beta\gamma}$ and produce cAMP levels which may then stimulate cAMP-responsive transcription.


hormones/neurotransmitters
R_q
$\beta\gamma$
αq
$\beta\gamma$
αs
R_s
R_i
αi
$\beta\gamma$
(+)
PKC
(+)
(+)
(+)
(+)
(+)
AC2
cAMP
AC4
(+)
ATP
PKA
ATP
(+)
CREB
CRE
DNA

CHAPTER V. WORKS IN PROGRESS AND FUTURE DIRECTIONS

Introduction: Oscillations of Ca^{2+}/CaM-stimulated adenylyl cyclase activity in pineal gland: implications for melatonin biosynthesis

The circadian organization of behavior affects how organisms respond to daily light/dark cues as well as longer-term seasonal changes changes in day length. In humans, disruption of the circadian cycle can lead to mental fatigue, sleep disorders, and depression (Moore, 1996). In brain, an endogenous circadian pacemaker resides in the suprachiasmatic nucleus of the hypothalamus (SCN) [Ralph, 1990 #192. Light input to the retina is relayed via the retinohypothalamic tract to the SCN (Hendrickson et al., 1972; Moore, 1973; Moore and Lenn, 1972)], where glutamate release modulates the circadian pacemaker (Castel et al., 1993; Rea et al., 1993). Several other hormones including serotonin (Lovenberg et al., 1993; Prosser et al., 1990), neuropeptide Y (Shibata and Moore, 1993), and melatonin (Sumova and Illnerova, 1996) can also impinge upon SCN pacemaker function. Of these, melatonin has recently received tremendous attention for its potential as a treatment for circadian clock dysfunction in humans (Tang et al., 1996).

Melatonin biosynthesis occurs in both the pineal gland (Korf, 1994) and the retina (Cahill et al., 1991) during the dark phase of the light/dark cycle. Pineal melatonin is thought to play a feedback role in entraining the SCN clock to environmental light/dark status (Moore, 1996; Redman et al., 1983), as well as affecting a wide variety of physiological processes in target tissues on a circadian basis (Tang et al., 1996). In retina, melatonin may regulate membrane turnover in rod outer segments (Besharse and Dunis, 1983), cone photoreceptor retinomotor movements (Pierce and Besharse, 1985; Pierce and Besharse, 1987), and aggregation of melanin pigments in the retinal pigment epithelium (Cheze and Ali, 1976; Kraus-Ruppert and Lembeck, 1965; Pang and Yew, 1979).

Melatonin is synthesized at night from serotonin by the consecutive actions of arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) (Cahill et al., 1991; Klein, 1985). AA-NAT is the rate-limiting enzyme for melatonin biosynthesis and its activity exhibits a dramatic circadian oscillation in both pineal (Klein and Weller, 1970) and retina (Binkley et al., 1979; Hamm and Menaker, 1980), where AA-NAT activity is greatly elevated during the dark phase of the light/dark cycle. The nocturnal increase in AA-NAT activity in both pineal and retina is due to cAMP-stimulated transcription of the AA-NAT gene (Baler et al., 1997; Borjigin et al., 1995; Roseboom et al., 1996; Roseboom and Klein, 1995), as well as cAMP-dependent posttranslational mechanisms which may maintain the enzyme in a highly activated state. In pineal, nocturnal norepinephrine (NE) release from superior cervical ganglion fibers binds to both α_1- and β-adrenergic receptors in the pineal, resulting in robust increases in cAMP levels (Vanacek et al., 1985). Although the relative contributions of the α_1 and β-adrenergic signals to induction of the AA-NAT gene are not clear, the possibility that AA-NAT gene induction occurs primarily or solely through the cAMP pathway is plausible since the type I adenylyl cyclase (designated AC1) is synergistically stimulated by paired Ca^{2+} and G_S signals (Wayman et al., 1994). In addition, AC1 can mediate synergistic induction of cAMP-responsive transcription by Ca^{2+} and G_S-coupled receptors (Impey et al., 1994).

Interestingly, Tzavara et al have recently reported the existence of a circadian oscillation of the AC1 mRNA in rat pineal, as well as substantial increases in rat pineal Ca^{2+}/CaM-stimulated adenylyl cyclase activity during the dark phase of the light/dark cycle (Tzavara et al., 1996). The oscillation of AC1 mRNA in pineal was dependent on NE input from the superior cervical ganglion. Since our laboratory created a mouse strain which lacks AC1, we are in an ideal position to determine if the oscillation of pineal Ca^{2+}/CaM-stimulated adenylyl cyclase activity is due to AC1. To accomplish this goal in

mice, it is necessary to demonstrate that the circadian rhythm of pineal Ca^{2+}-stimulated adenylyl cyclase activity occurs in mice. Since retina also possesses an intrinsic, oscillatory melatonin synthetic pathway (Binkley et al., 1979; Cahill et al., 1991; Hamm and Menaker, 1980), as well as AC1 expression (Xia et al., 1993), an additional goal of this study was to determine if Ca^{2+}/CaM-stimulated adenylyl cyclase activity exhibited a circadian oscillation in retina. Using AC1 knockout mice, we are in the process of testing whether disruption of the gene for AC1, vis a vis potential regulation of melatonin biosynthesis, had substantial effects on a circadian behavioral output, in this case locomotor activity. To this point, I have demonstrated a circadian oscillation of wild-type mouse pineal Ca^{2+}/CaM-stimulated adenylyl cyclase activity, consistent with previous results in rat (Tzavara et al., 1996). I have observed that Ca^{2+}/CaM-stimulated adenylyl cyclase activity does not oscillate in mouse eye cup membrane preparations. Additionally, the circadian locomotor activity of AC1 mutant mice may be altered relative to wild-type mice. Finally, although I have been unable to demonstrate that the oscillation of pineal Ca^{2+}/CaM-stimulated adenylyl cyclase activity is due to AC1, these experiments are of high priority and will be attempted again shortly.

Materials and Methods

Materials- Calmodulin was purified from bovine brain according to the method of LaPorte (LaPorte and Storm, 1978). [^{32}P]-α-ATP was from ICN (Costa Mesa, CA).

Cell Culture- Human embryonic kidney 293 (HEK 293) cells were grown at 37°C in HEPES-buffered Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% bovine calf serum (BCS) in a humidified 95% air/5% CO_2 incubator. Cell culture

materials were from Life Technologies, Inc. (Gaithersburg, MD) unless otherwise indicated.

Expression of AC1 in HEK 293 Cells- The AC1 cDNA clone was isolated from a bovine brain cDNA library as described (Xia et al., 1991). The AC1 insert was subcloned into the pCEP4 expression vector (Invitrogen, San Diego, CA). Polyclonal populations of hygromycin (Calbiochem, La Jolla, CA; 500units/ml)-resistant HEK 293 cells were obtained by stable transfection of pCEP4 or pCEP4-AC1 using the calcum phosphate method (Chen and Okayama, 1987). Expression of transfected AC1 was determined by cAMP accumulation assays as described in Wayman *et al* (Wayman et al., 1994).

Animals- 8-12 week old 129JR2448 mice of both sexes were used. Animals were maintained on a 12/12 light/dark cycle (lights on at 0600) and adapted to this rhythm for at least one week prior to experimental manipulations. For pineal dissections, animals were decapitated, pineals were isolated, and frozen on dry ice. Daytime dissections were done between 11 a.m. and 1 p.m. and nighttime dissections were done between 11 p.m. and 1 a.m. For eye cup preparations, eyes were removed and placed into ice-cold PBS for 15 minutes. Using a scalpel, a vertical slit was made at the front of the eye and the lens and humor were carefully removed through the slit. All tissue was frozen at -80^{o}C until use (<1 week). For nighttime dissections, animals were killed under dim red light.

Adenylyl Cyclase Assay- In general, adenylyl cyclase assays were carried out as described in Villacres et al (Villacres et al., 1995). For pineal assays, tissue was thawed in ice-cold homogenization buffer (20mM Tris-HCl pH 7.4, 2mM $MgCl_2$, 1mM EDTA, 0.5mM DTT, 0.5mM PMSF, 3.2μg/ml leupeptin, 2μg/ml aprotinin). For each experimental

condition (day/night), the pineal glands of 8-10 mice were used. Pineal membranes were prepared by Dounce homogenization (30 strokes) of dissected tissue and centrifugation at 735x*g* for 5 minutes with retention of the supernatant. The supernatant was centrifuged at 40,000x*g* in a Beckman ultracentrifuge. Membrane pellets were resuspended in homogenization buffer and assayed. To determine Ca^{2+}/CaM-stimulated adenylyl cyclase activity, assays included 2.4μM CaM and 677nM free Ca^{2+}. cAMP was quantified according to the method of Salomon (Salomon et al., 1974).

For eye cup assays, dissected tissue was thawed, resuspended in ~10 volumes of ice-cold homogenization buffer, and disrupted with a hand-held polytron for 15 seconds. Membranes were isolated and assayed as described above for pineals. For Ca^{2+} dose-responses, free Ca^{2+} concentrations were estimated using the Bound and Determined computer software (Brooks and Storey, 1992).

Monitoring of Mouse Circadian Locomotor Activity - To measure the locomotor activity of wild-type and AC1 knockout mice, mice were housed individually in cages affixed with an infrared motion detector (Mini-Mitter Co., Inc., Sunriver, OR) on the top of the cage. Mice were adapted to the presence of the motion detector for 2-3 days prior to collection of activity data. The number of infrared beam breaks per 10 minute sampling interval for each mouse was collected by a PC running Vital View software (Mini-Mitter). Data was analyzed using Vital View software.

Results and Discussion

Ca^{2+}/CaM-stimulated adenylyl cyclase activity does not undergo a circadian oscillation in mouse retina- Retina and pineal are related both embryologically and biochemically (Korf, 1994). More specifically, both retina and pineal exhibit rhythmic melatonin production,

cAMP-dependent induction of AA-NAT on a circadian basis, and expression of AC1. Since the mRNA for AC1 undergoes a circadian oscillation in rat pineal, we tested whether there was a difference in mouse retinal Ca^{2+}/CaM-sensitive adenylyl cyclase activity between tissue collected during the day or at night. We found that there was no substantial difference in Ca^{2+}/CaM-stimulated adenylyl cyclase activity between the day or night (Figure 32). At 677nM free Ca^{2+}, mouse eye cup Ca^{2+}/CaM-sensitive adenylyl cyclase isolated during the day or at night was stimulated ~2.5-3-fold. This suggests that mouse retinal Ca^{2+}/CaM-stimulated adenylyl cyclase activity does not undergo a circadian oscillation, however it may be necessary to examine time points in addition to noon and midnight.

AC1 is the major Ca^{2+}/CaM-stimulated adenylyl cyclase in mouse retina- It was of interest to compare the Ca^{2+} sensitivity of wild-type and AC1 mutant mouse eye cup adenylyl cyclase activity. In addition, we tested whether or not there were any substantial differences in absolute activity or Ca^{2+} sensitivity between samples collected during the day or night from both sets of mice. I demonstrated that Ca^{2+}/CaM-stimulated adenylyl cyclase activity is decreased by ~55-60% in AC1 mutant mice in both day and night samples (Figures 33A and 33B). The EC50 for Ca^{2+} in wild-type membranes was ~250nM during the day and ~150nM at night. In the mutant mice, Ca^{2+} EC50's were ~650nM during the day and ~500nM at night. The lower Ca^{2+} sensitivity in the mutant mice is consistent with that of AC8 (Villacres et al., 1995). Interestingly, adenylyl cyclase activity in both wild-type and mutant mice eye cups was quite similar at both day and night time points, indicating the absence of a circadian oscillation of AC1 or some other Ca^{2+}-stimuated adenylyl cyclase. Since melatonin exhibits a circadian oscillation in retina, it is intriguing that there is no oscillation of Ca^{2+}/CaM-stimulated adenylyl cyclase in retina, while there is in pineal (Tzavara et al., 1996). It is possible that some other adenylyl

cyclase is oscillating, however, the constitutive presence of AC1 in retina could produce an oscillatory cAMP signal, since Ca^{2+} concentrations in photoreceptor cells are higher in the dark than in light (Grey-Keller and Detwiler, 1994). Therefore, differences in the concentration of calcium between day and night may contribute to an oscillatory cAMP signal mediated by AC1 which generates a circadian rhythm of AA-NAT activity in retina (Figure 39).

To further characterize the Ca^{2+}/CaM-stimulated adenylyl cyclase activity in wild-type mouse eye, we compared the Ca^{2+} sensitivity of the eye cup adenylyl cyclase to AC1 expressed in HEK 293 cells. HEK 293 cells do not express Ca^{2+}/CaM-stimulated adenylyl cyclases, therefore, the Ca^{2+} sensitivity of AC1 in HEK 293 cells is an accurate readout of its Ca2+ sensitivity. We observed that wild-type mouse retinal Ca^{2+}/CaM-stimulated adenylyl cyclase activity was quite similar to AC1 expressed in HEK 293 cells (Figure 34). The EC50 for Ca^{2+} in retina was ~300 nM, while that for AC1 in HEK 293 cells was ~150nM, in strong agreement with previously reported values (Villacres et al., 1995). This suggests that AC1 is the major retinal Ca^{2+}/CaM-stimulated adenylyl cyclase, but there is a contribution of one or more Ca^{2+}-stimulated adenylyl cyclases with lower Ca^{2+} sensitivity than AC1.

Ca^{2+}/CaM-stimulated adenylyl cyclase activity undergoes a circadian oscillation in mouse pineal gland- Next, we sought to determine if there was a circadian fluctuation in mouse pineal Ca^{2+}/CaM-stimulated adenylyl cyclase activity. We isolated pineals from 8-10 mice during the day and at night and assayed them for Ca^{2+}-stimulated adenylyl cyclase activity. We found that there was was a substantial increase in Ca^{2+}/CaM-stimulated adenylyl cyclase activity at night relative to day (Figure 35). The fold-stimulation of mouse pineal adenylyl cyclase by Ca^{2+}/CaM was elevated ~200% at night relative to the daytime. This

result is consistent with the report of Tzavara et al (Tzavara et al., 1996) demonstrating an AC1 oscillation in rat pineal.

AC1 mutant mice exhibit differences in circadian locomotor activity when compared to wild-type mice- To address the physiological consequences of the AC1 gene disruption on circadian behavioral output, I monitored the locomotor activity of wild-type and AC1 mutant mice (Figure 36). Nocturnal animals have their active periods during the dark phase of light dark cycle. Although, more trials and analysis of locomotor activity will certainly be required, my initial efforts at monitoring the locomotor activity of wild-type and AC1 mutant mice are described at the end of Chapter V. I have found that both the wild-type and AC1 knockout mice demonstrate circadian oscillations of locomotor activity, with activity being highest during the dark phase. During a normal 12/12 (L/D) cycle, there was a subtle difference between groupsduring periods of relative inactivity in the light phase. The wild-type mice tended to show a "troughing" in their activity profiles. The were only inactive for a short period of time and they showed light activity before and after the brief period of inactivity. For the mutant mice, these same periods of inactivity were characterized by almost complete inactivity. The significance of this result has yet to be determined.

A second intriguing difference between the activity profiles of the wild-type and mutant mice was uncovered when I changed the light dark cycle to 6 hours light/18 hours dark. Typically, when animals are phase-shifted, they eventually adapt to the new rhythm of light/dark changes. I observed that the wild-type mice did adapt to the phase-shift in terms of locomotor activity. At the new time of dark onset, the wild-type mice increase their locomotor activity. On the other hand, the AC1 mutant mice maintained their previous 12/12 (L/D) cycle with regards to activity (Figure 37). This indicates that AC1 may be important for animals' adaptability to changes in day/night length. If melatonin regulates

circadian locomotor activity, the AC1 gene disruption may affect an animal's ability to adapt to changes in the light/dark cycle by compromising pineal melatonin synthesis. Clearly, more experimentation will be required to elucidate the role of AC1, melatonin, and how they relate to a circadian output such as locomotor activity. In any case, the results described here are intriguing and suggest that AC1 may play a substantial role in regulating circadian rhythms.

At this time, I have been unable to demonstrate that the rhythm in Ca^{2+}/CaM-stimulated adenylyl cyclase activity in mouse pineal is due to AC1. However, the results obtained to this point illustrate some potentially interesting differences between retina and pineal as far as the mechanism for generating a circadian rhythm of AA-NAT transcription and activity. The pineal may accomplish its NAT rhythm by synthesizing and degrading a critical protein (AC1) for establishing the rhythm (Figure 38), while retina may rely on differences in the photoreceptor cells' biophysical and neurochemical properties to diurnally regulate the activity of the same critical protein (AC1) (Figure 39). Utilizing AC1 mutant mice, it will be extremely intersting to address the question of whether the circadian rhythm of NAT activity, and thus, melatonin biosynthesis, is compromised. If this is the case, there are a large number of melatonin-regulated physiological processes (Tang et al., 1996) which may be affected in the AC1 knockout mice.

Figure 32. Ca^{2+}/CaM-stimulated adenylyl cyclase activity does not exhibit a circadian oscillation in mouse retina. Membranes prepared from mouse eye cup were isolated at noon (day) or midnight (night) and assayed for Ca^{2+}/CaM-sensitive adenylyl cyclase activity. Free Ca^{2+} was 677nM. Adenylyl cyclase assays were carried out as described under "Materials and Methods". The data are the means +/- S.D. of triplicate assays.

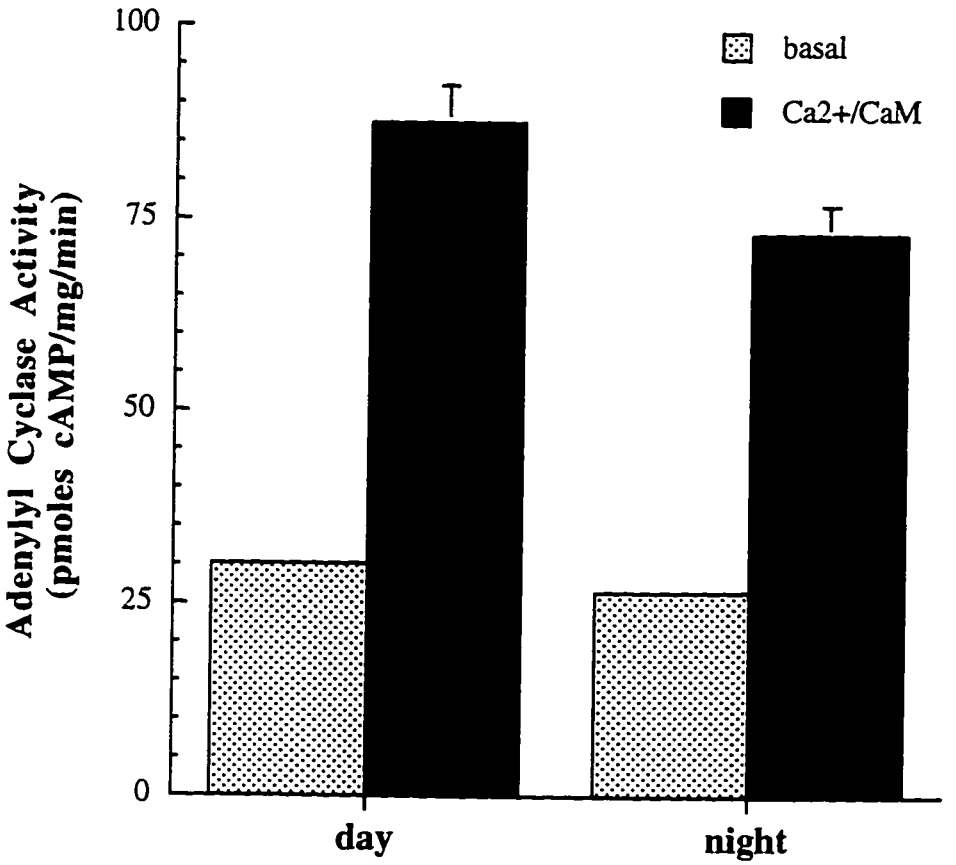
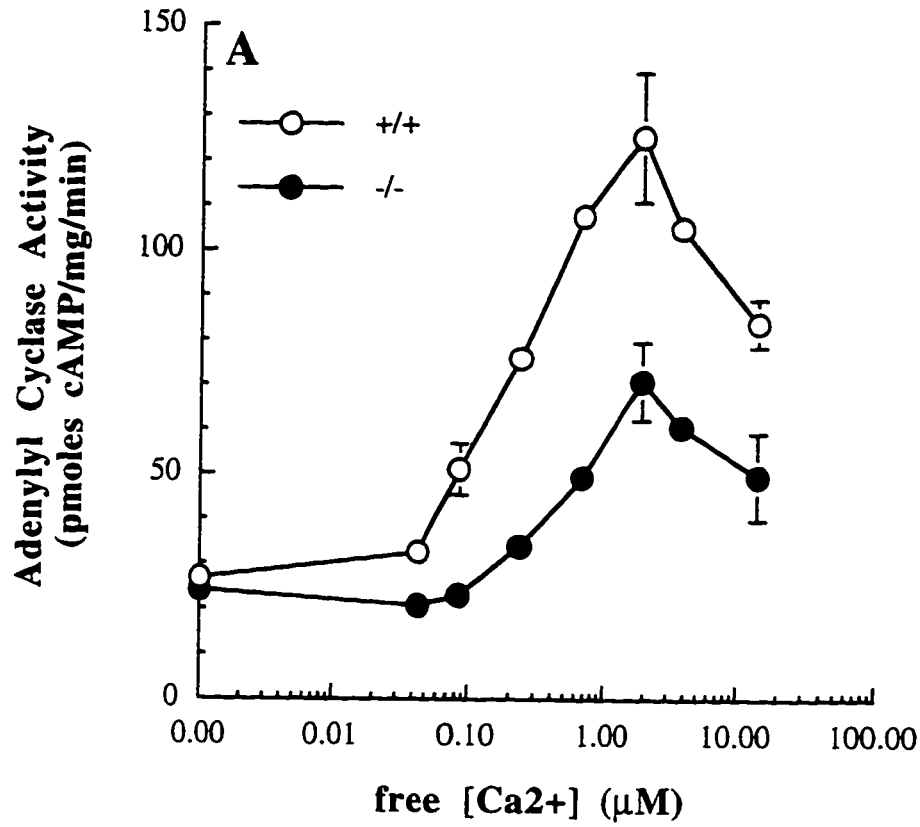

Adenylyl Cyclase Activity
(pmoles cAMP/mg/min)
100
75
50
25
0
basal
Ca2+/CaM
day
night

Figure 33. Ca^{2+}/CaM-stimulated adenylyl cyclase activity is reduced in membranes from AC1 mutant mouse eye cups. Membranes were prepared from wild-type (+/+) or AC1 knockout mouse (-/-) eye cups isolated at noon (A) or midnight (B). Membranes were assayed for Ca^{2+}/CaM-sensitive adenylyl cyclase activity at various free Ca^{2+} concentrations as described under "Materials and Methods". The data are the means +/- S.D. of triplicate assays. Free Ca^{2+} concentrations were estimated using Bound and Determined computer software.


A
+/+
-/-
150
100
50
0
0.00
0.01
0.10
1.00
10.00
100.00
Adenylyl Cyclase Activity
(pmoles cAMP/mg/min)
free [Ca2+] (μM)

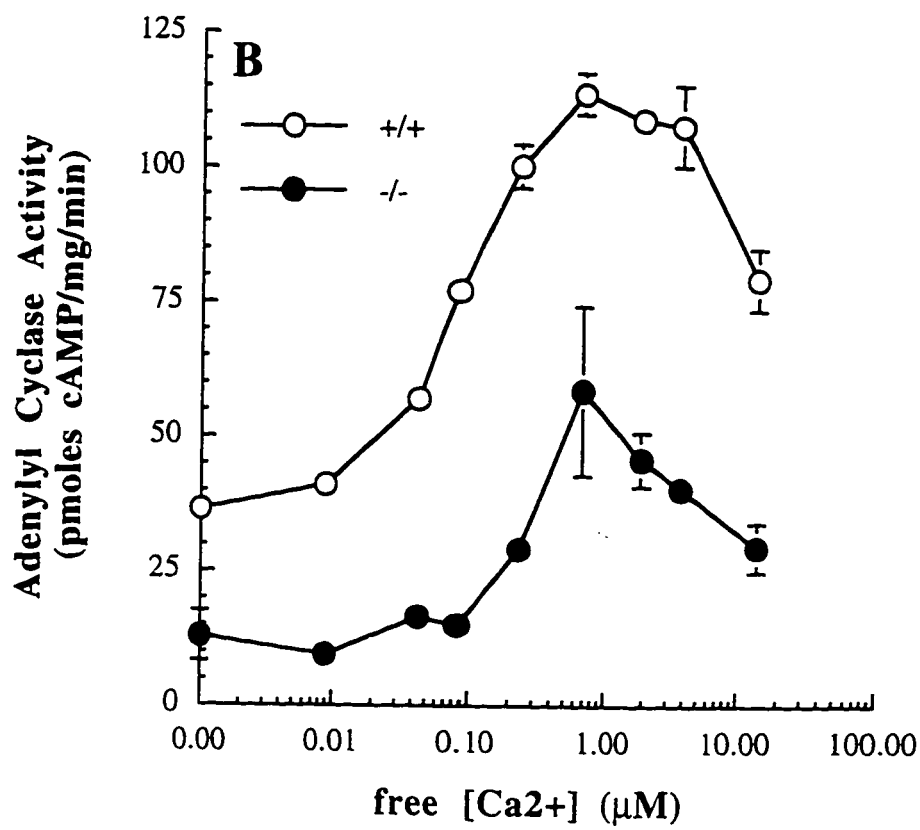

B
+/+
-/-
125
100
75
50
25
0
0.00
0.01
0.10
1.00
10.00
100.00
Adenylyl Cyclase Activity
(pmoles cAMP/mg/min)
free [Ca2+] (μM)

Figure 34. Comparison of wild-type mouse eye cup Ca^{2+}/CaM-stimulated adenylyl cyclase activity to AC1 expressed in HEK 293 cells. Membranes from mouse eye cup (isolated during the day) or HEK 293 cells expressing AC1 (designated 293-AC1) were assayed for Ca^{2+}/CaM-sensitive adenylyl cyclases in the presence of various free Ca^{2+} concentrations as described under "Materials and Methods". The data are the means +/- S.D. of triplicate assays. Free Ca^{2+} concentrations were estimated using Bound and Determined computer software.

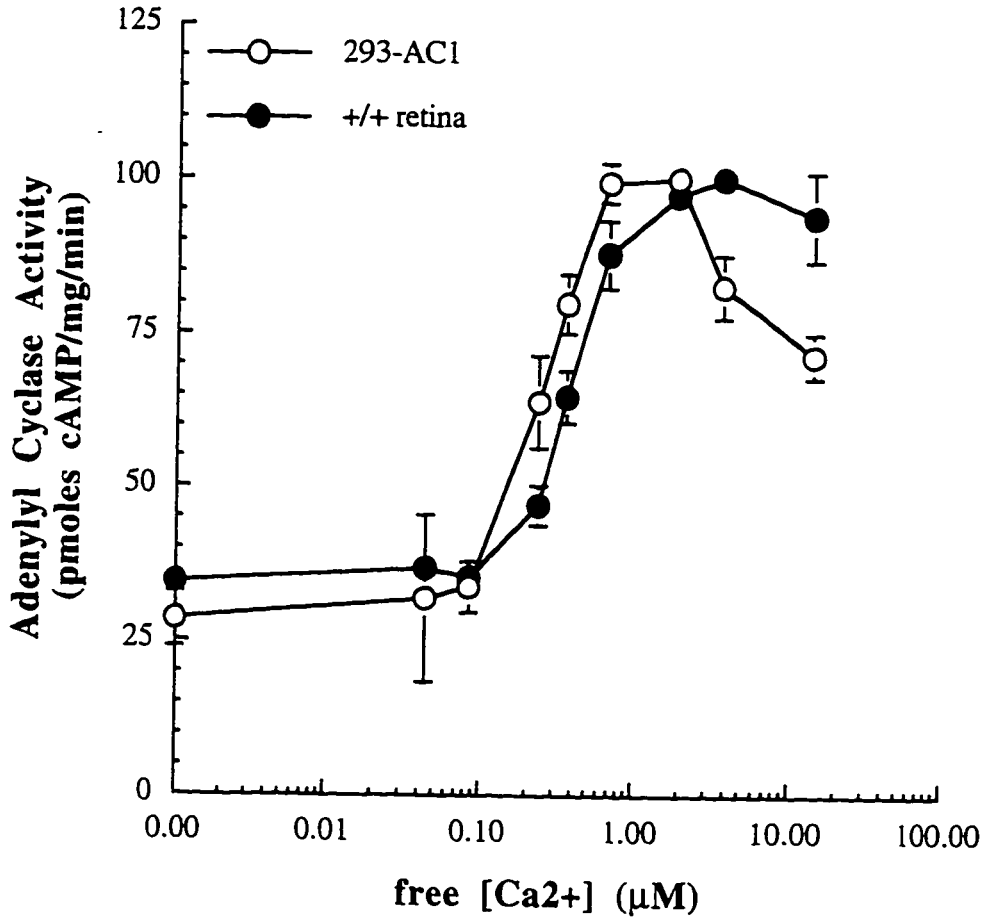

125
100
75
50
25
0
293-AC1
+/+ retina
Adenylyl Cyclase Activity
(pmoles cAMP/mg/min)
0.00
0.01
0.10
1.00
10.00
100.00
free [Ca2+] (μM)

Figure 35. Ca^{2+}/CaM-stimulated adenylyl cyclase activity exhibits a circadian oscillation in mouse pineal gland. Mouse pineal glands were isolated at noon (day) or midnight (night). Pineal membranes were assayed for Ca^{2+}/CaM-sensitive adenylyl cyclase activity as described under "Materials and Methods". The free Ca^{2+} concentration was 677nM. The data are the means +/- S.D. of triplicate assays. Free Ca^{2+} concentrations were estimated using Bound and Determined computer software.

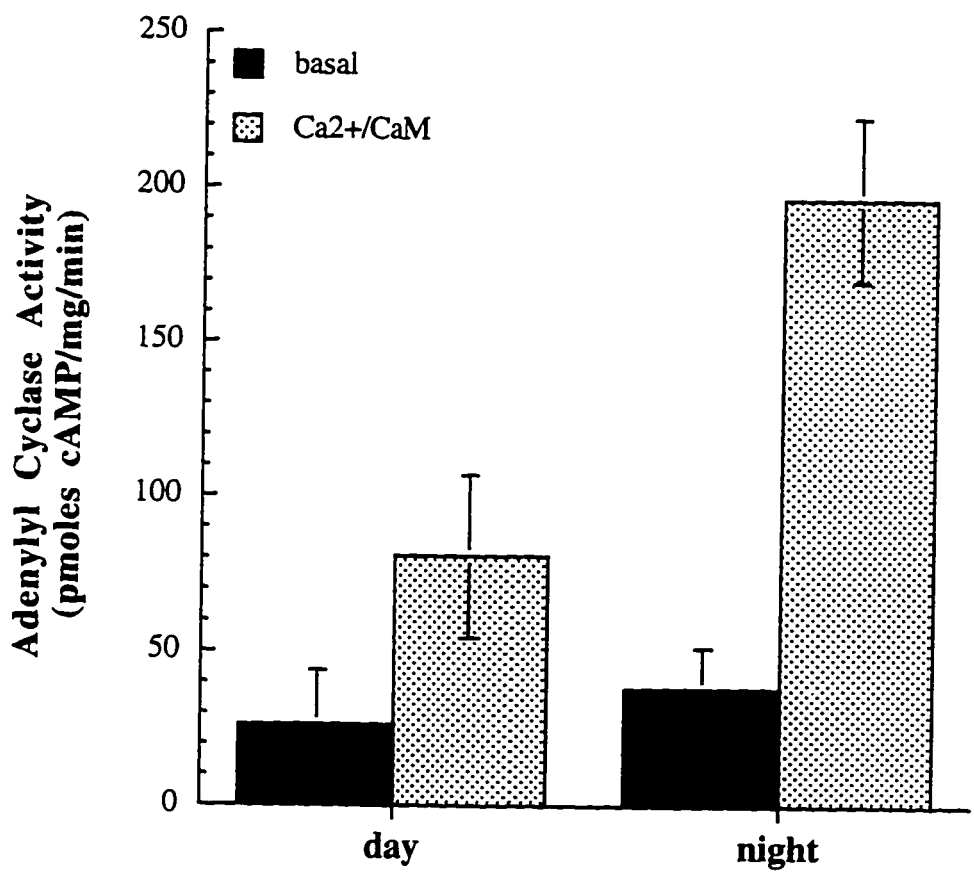

Adenylyl Cyclase Activity
(pmoles cAMP/mg/min)
250
200
150
100
50
0
basal
Ca2+/CaM
day
night

Figure 36. Locomotor activity profile of wild-type and AC1 knockout mice. Locomotor activity was continuously monitored as described under "Materials and Methods". Mice were adapted to the light/dark cycle and the presence of the infrared detectors for 2-3 days prior to collection of data. Data was collected for three days with the mice on a 12/12 (L/D) cycle. During the light phase on day 4, the L/D cycle was changed to 6/18 (L/D), and this was maintained for 5 days. On day 9, the cycle was shifted back to the original 12/12 (L/D). Wild-type mice are represented on the upper panel and AC1 knockouts are represented on the lower panel. Data are the group means (n=8) for each 10 minute sampling interval throughout the data acquisition period.

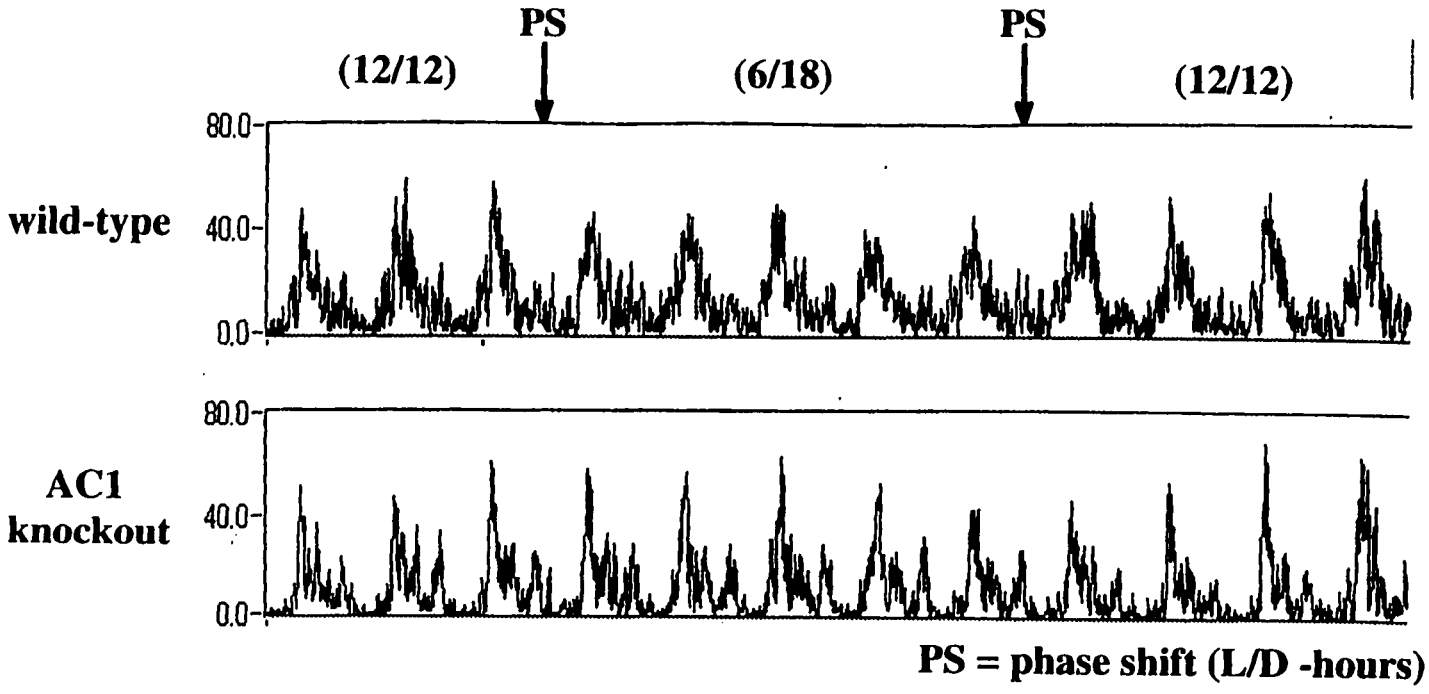

PS
PS
(12/12)
(6/18)
(12/12)
wild-type
80.0
40.0
0.0
AC1
knockout
80.0
40.0
0.0
PS = phase shift (L/D -hours)

Figure 37. AC1 knockout mice do not become entrained to a shift of the light/dark cycle. The data from Figure 36 has been "zoomed" to focus on days 8-10. "On" and "off" refers to the room lights. Data are the group means (n=8) for each 10 minute sampling interval during the monitoring period shown.

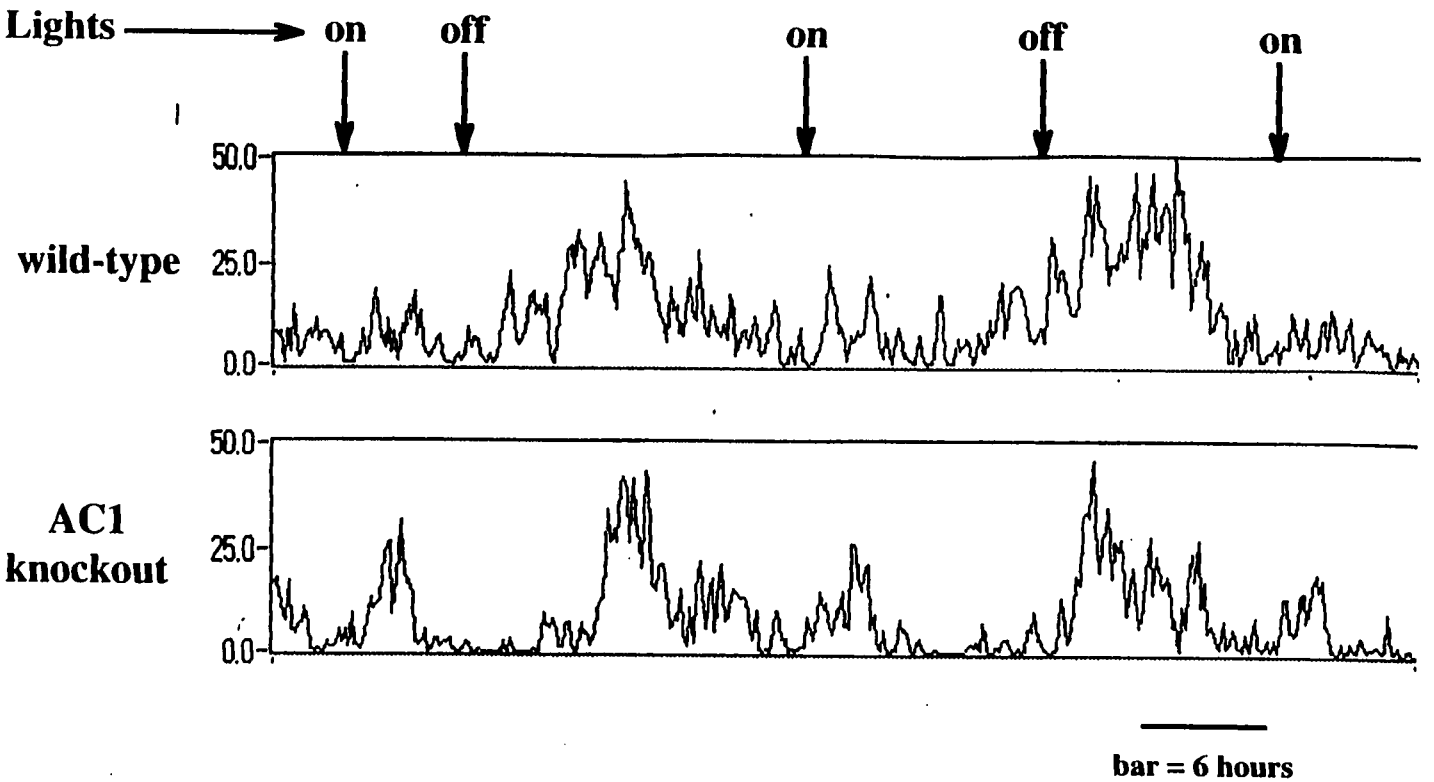

Lights
on
off
on
off
on
50.0
25.0
0.0
wild-type
50.0
25.0
0.0
AC1
knockout
bar = 6 hours

Figure 38. A diurnal rhythm of AC1 may contribute to circadian melatonin biosynthesis in pineal gland. During the night, NE release from the superior cervical ganglion (SCG) activates both α_1 andβ-adrenergic recptors in the pineal. During the night AC1 protein is expressed and generates large cAMP signals in response to paired G_S and Ca^{2+} signals. This stimulates CRE-mediated transcription of the AA-NAT gene, as well as posttranslationally increasing the activity of AA-NAT protein, and melatonin is synthesized. During the day, there is little NE release onto the pineal, or expression of AC1, therefore the cAMP-dependent transcriptional processes operative at night are inactive (i.e., AA-NAT transcription) and melatonin is not synthesized.

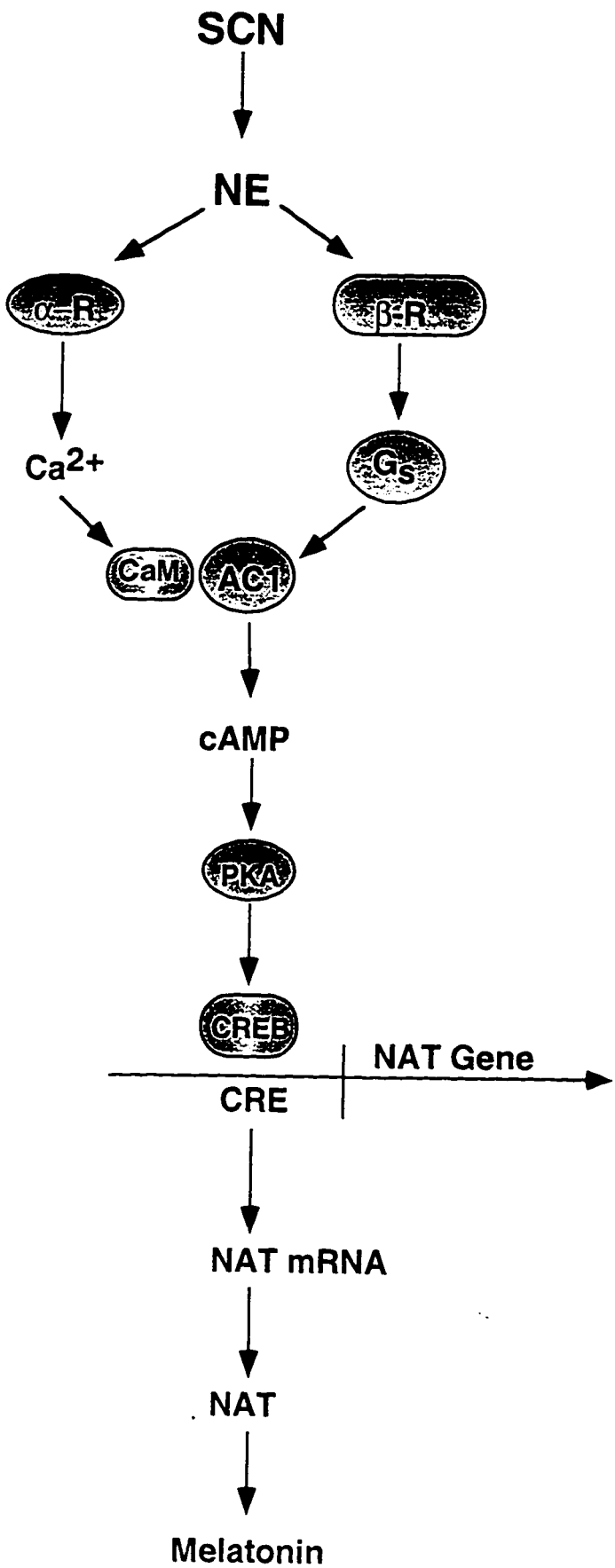
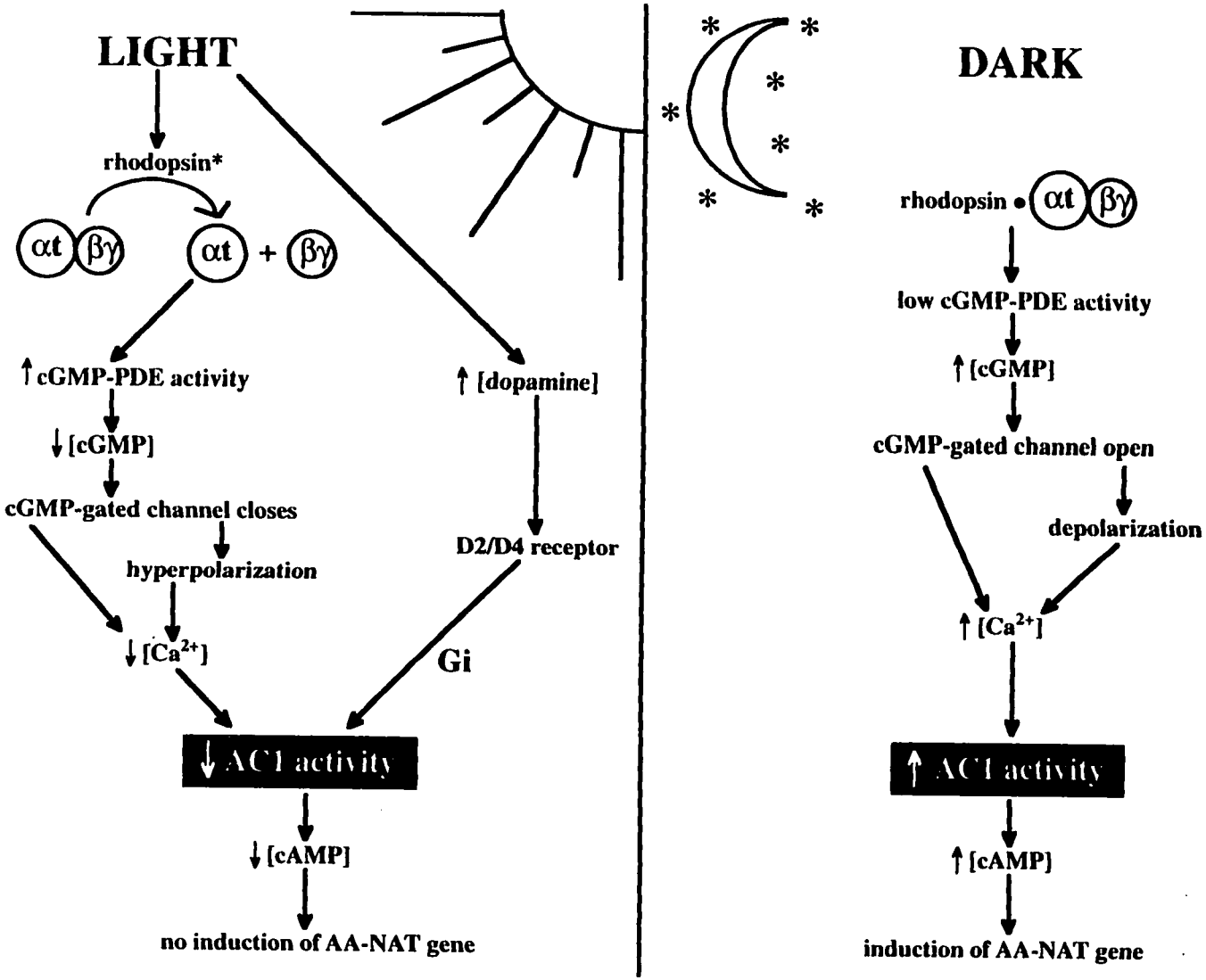

SCN
NE
α-R
β-R
Ca2+
Gs
CaM
AC1
cAMP
PKA
CREB
NAT Gene
CRE
NAT mRNA
NAT
Melatonin

Figure 39. Rhythmic melatonin synthesis in mouse retina does not require an oscillation of AC1 expression levels. During the light phase (left panel), light input to the retina activates a cGMP phosphodiesterase via the retinal G protein transducin (α_t·$\beta\gamma$) and cGMP levels decrease. Lowered levels of cGMP decrease Ca^{2+} flux through cGMP-gated ion channels, leading to hyperpolarization of the cell. Hyperpolarization also decreases Ca^{2+} entry through voltage-gated Ca^{2+} channels such that, during the day, intracellular Ca^{2+} is low and AC1 is relatively inactive. With low cAMP levels, the AA-NAT gene is not transcribed. During the dark phase (right panel), cGMP levels rise and photoreceptor cells become depolarized, both of which lead to increases of intracellular Ca^{2+}. Ca^{2+}/CaM activates AC1 and cAMP levels rise. Elevated cAMP levels lead to activation of AA-NAT gene transcription and, thus, melatonin synthesis.


LIGHT
rhodopsin*
αt
βγ
αt + βγ
↑cGMP-PDE activity
↓[cGMP]
cGMP-gated channel closes
hyperpolarization
↓[Ca2+]
↑ [dopamine]
D2/D4 receptor
Gi
↓ AC1 activity
↓[cAMP]
no induction of AA-NAT gene
DARK
rhodopsin • αt βγ
low cGMP-PDE activity
↑[cGMP]
cGMP-gated channel open
depolarization
↑[Ca2+]
↑ AC1 activity
↑[cAMP]
induction of AA-NAT gene

CHAPTER VI. CONCLUSIONS

The underlying biochemical mechanisms which contribute to long-term changes in synaptic strength in the hippocampus are beginning to be understood (Fagnou and Tuchek, 1995). The cAMP signal transduction pathway plays a role in several forms of synaptic plasticity in the mammalian hippocampal formation (Frey et al., 1993; Gereau IV and Conn, 1994; Huang et al., 1995; Huang et al., 1994; Impey, 1996; Weisskopf et al., 1994; Wu et al., 1995). In particular, at the mf-CA3 synapse, application of cAMP analogs or the adenylyl cyclase activator forskolin produces LTP (Huang et al., 1994; Weisskopf et al., 1994). Knockout mice lacking AC1 or the a RIβ isoform of PKA show compromised mossy fiber-CA3 LTP ((Huang et al., 1995), Villacres and Storm, unpublished observations) . In CA1, L-LTP depends on elevations of cAMP (Frey et al., 1993), and these cAMP increases activate CRE-mediated transcription (Impey, 1996). Since cAMP plays a role in several forms of synaptic plasticity in the hippocampus, it is necessary to determine which isoforms of adenylyl cyclase are expressed in the hippocampus, as well as elucidating the regulatory properties of these adenylyl cyclases in intact cells (see Table IV). These represent the goals of this thesis.

Work in Chapter II focused on the inhibitory regulation of the Ca^{2+}/CaM-stimulated adenylyl cyclases AC1 and AC8 expressed in HEK 293 cells. Both AC1 and AC8 are expressed in the hippocampus and coupling of the Ca^{2+} and cAMP signalling pathways is thought to be an important biochemical correlate underlying long-term adaptive changes in neurons (Abrams et al., 1991; Feany, 1990; Levin et al., 1992; Livingston et al., 1984; Xia et al., 1995). However, since optimal, not necessarily maximal, levels of cAMP may be necessary for learning and memory (Feany, 1990), inhibition of Ca^{2+}-stimulated adenylyl cyclases may be equally important. Furthermore, since pertussis toxin inhibits the development of LTP in several areas of hippocampus (Goh and Pennefather,

1990; Goh and Pennefather, 1989; Ito et al., 1988), a role for inhibitory G proteins in synaptic plasticity is suggested.

I found that AC1, but not AC8, was inhibited by G_i-coupled somatostatin and dopamine D2L receptors in HEK 293 cells. Since both α_i and $\beta\gamma$ inhibit AC1 *in vitro* (Tang et al., 1991; Tang and G., 1991; Taussig et al., 1993; Taussig et al., 1994; Tota et al., 1990), it was of interest to determine how G_i-coupled receptor activation elicits inhibition of AC1 *in vivo*- is it due to α_i, $\beta\gamma$, or both? I found that inhibition of Ca^{2+}-stimulated AC1 was primarily due to α_i and not $\beta\gamma$ since coexpression of the $\beta\gamma$ scavengers βARK_1-ct or transducin-α did not affect inhibition of AC1 by G_i-coupled receptors. Therefore, the primary mechanism for inhibition of AC1 by G_i-coupled receptors is through α_i.

A second aspect of this study was to determine if $\beta\gamma$ release from G_S prevents G_S-coupled receptor stimulation of AC1 *in vivo*. AC1 is insensitive to G_S-coupled receptor activation unless intracellular Ca^{2+} is elevated and CaM is bound to the enzyme (Wayman et al., 1994). I demonstrated that sequestration of $\beta\gamma$ in intact cells elicits stimulation of AC1 by the G_S-coupled 5HT7 receptor. This observation suggests that $\beta\gamma$ release from G_S inhibits α_S stimulation of AC1.

The results in Chapter II, as well as those detailed elsewhere (Villacres et al., 1995; Wayman et al., 1996), indicate that AC1 and AC8 are not redundant enzymatic activities (see Table III). These enzymes have evolved with specific regulatory properties which underlie their functional roles, perhaps during synaptic plasticity. I hypothesize that AC1 may be important for processes which require transient increases of cAMP since its activity is attenuated by G_i-coupled receptors and CaM kinase IV (Wei, 1996). At the mossy fiber-CA3 synapse during LTP, it has been hypothesized that Ca^{2+} coupling to cAMP synthesis in mossy fiber terminals activates PKA and produces a persistent enhancement of glutamate

release, leading to LTP (Weisskopf et al., 1994). In CA1, L-LTP depends cAMP (Frey et al., 1993), and stimuli which produce L-LTP also stimulate cAMP-responsive transcription (Impey, 1996). Since activation of CRE-mediated transcription may require prolonged increases of cAMP (Hagiwara et al., 1993), the insensitivity of AC8 to inhibition by G_i-coupled receptors and CaM kinases suggest that Ca^{2+} stimulation of AC8 may provide a prolonged cAMP signal. Taken together, differential inhibition of the Ca^{2+}/CaM-stimulated adenylyl cyclases by G_i-coupled receptors may be an important component to the functional roles of AC1 and AC8 during synaptic plasticity in the mammalian hippocampus.

In brain, mRNA expression of the G_s-coupled $5HT_6$ and $5HT_7$ receptors overlaps those of several adenylyl cyclases. I evaluated coupling between $5HT_6$ and $5HT_7$ with the G_s-sensitive AC5, as well as with the Ca^{2+}/CaM-stimulated AC1 and AC8. I observed that each receptor stimulated AC5, as predicted. Unexpectedly, $5HT_7$, but not $5HT_6$, stimulated AC1 and AC8. $5HT_7$ stimulation of AC1 and AC8 was dependent on Ca^{2+} since Ca^{2+} depletion or chelation treatments substantially inhibited $5HT_7$ stimulation of the Ca^{2+}-stimulated adenylyl cyclases AC1 and AC8. Of significance is that activation of $5HT_7$ receptors has not been previously shown to elicit rises of intracellular Ca^{2+}. Although the mechanism for $5HT_7$ increases of intracellular Ca^{2+} has not been elucidated, future studies utilizing Fura-2 Ca^{2+} imaging will address this issue.

The observation that $5HT_7$ can stimulate AC1 and AC8 in a Ca^{2+}-dependent manner suggests that, in brain regions such as hippocampus where $5HT_7$ (Shen et al., 1993) and both AC1(Xia et al., 1991) and AC8 (Cali et al., 1994) are expressed, 5HT can modulate the Ca^{2+} and cAMP signal transduction pathways via activation of the Ca^{2+}/CaM-stimulated adenylyl cyclases AC1 and AC8. A role for 5HT in synpatic plasticity (in mammals) has not been clearly defined, although most of the 5HT receptor subtypes are expressed in hippocampus (Hoyer et al., 1994). Furthermore, there is a

strong serotonergic projection originating in the midbrain raphe nuclei which terminates in the hippocampus. Thus, a possible role for 5HT in hippocampal function (e.g., spatial learning) is not out of the question. A similar Ca^{2+}-dependent, 5HT7 stimulation of AC8 may be relevant in the hypothalamus and influence neuroendocrine function.

In the context of neuropsychiatric disorders, the coupling of $5HT_6$ and AC5 may be of relevance to the mechanism of action of antipsychotic drugs. $5HT_6$ is a siginificant target of many clinically useful psychotropic agents (Monsma et al., 1993; Roth et al., 1994). Since both $5HT_6$ and AC5 are highly expressed in the striatum/nucleus accumbens (Mons and Cooper, 1994; Ward et al., 1995), it is quite possible that these molecules are coupled *in vivo*, and that antipsychotic drugs such as clozapine may exert some of their effects through antagonism of $5HT_6$ stimulation of AC5 in these brain regions. These questions will be more amenable to experimental testing when specific ligands/antibodies become available to $5HT_6$ and AC5, as well as the possibility that AC5 knockout mice will be developed. In summary, work in Chapter 3 describes the coupling of the $5HT_6$ and 5HT7 receptors to AC1, AC5, and AC8. $5HT_6$ operates as a typical G_s-coupled receptor in that it stimulates AC5, but not AC1 or AC8. On the other hand, 5HT7 stimulates AC5, but also AC1 and AC8, the latter two of which was found to occur in a Ca^{2+}-dependent manner. This unexpected result strongly suggests that 5HT7 is capable of coupling to signal transduction pathways which elevate intracellular Ca^{2+}.

A second subfamily of adenylyl cyclases is comprised of AC2 and AC4. A unique feature of these two isoforms of adenylyl cyclase is that $\beta\gamma$ subunits released from G_i potentiate $G_{s\alpha}$-stimulated activities (Federman et al., 1992; Feinstein et al., 1991; Gao and Gilman, 1991; Lustig et al., 1993; Taussig et al., 1994; Taussig et al., 1993). In hippocampus, several researchers have observed that activation of G_i-coupled receptors potentiated cAMP increases caused by stimulation of G_s-coupled β-adrenergic receptors

(Andrade, 1993; Gereau IV and Conn, 1994). Treatments which elicited this G_i-mediated potentiation of G_s-stimulated cAMP levels also increased the excitability of CA1 pyramidal neurons, a phenomenon which may be important during synaptic plasticity. Interestingly, the regulatory properties of AC2 or AC4 may account for these results. Along these lines, mRNA for AC2 has been detected in hippocampus (Furuyama et al., 1993), however, there was no information regarding expression of AC4 in hippocampus. Due to the recent availability of antibodies to specific isoforms of adenylyl cyclase, I was very interested in determining the expression of the $\beta\gamma$-stimulated adenylyl cyclases AC2 and AC4 in hippocampus. Furthermore, I characterized the regulatory properties of AC4 in intact cells since they had not yet been elucidated.

Results in Chapter 4 demonstrate that the regulatory properties of AC4 *in vivo* parallel those determined *in vitro* (Gao and Gilman, 1991). That is, activation of G_s-coupled receptors stimulated AC4, and that stimulation was potentiated by somatostatin, a G_i-linked hormone. The potentiation of G_s-stimulated AC4 *in vivo* by somatostatin was blocked by pertussis toxin as well as coexpression of the $\beta\gamma$ scavengers βARK_1-ct and $G_{t\alpha}$. These results demonstrate that AC4, like AC2, responds to paired G_s and G_i signals with suprastimulation.

After determining that antibodies to AC2 and AC4 specifically recognized their respective protein antigens in intact cells, expression of AC2 and AC4 in hippocampus was elucidated. It was found by immunohistochemistry that both AC2 and AC4 were expressed in mouse hippocampus. AC2 was found primarily in dentate gyrus and CA1, with only weak labeling of CA3. Antibodies to AC4 stained dentate gyrus, CA3, and CA1. For both isoforms, labeling in dentate gyrus was observed in cell bodies and their associated processes. In CA1, labeling of pyramidal cell dendrites in the statum radiatum was prominent for both AC2 and AC4. In particular, AC2 staining occurred in a punctate manner along CA1 dendrites, with only minimal labeling of cell bodies. AC4 was found in

CA1 pyramidal cell bodies as well as in dendrites, where staining was quite intense. These results demonstrate that the βγ-stimulated adenylyl cyclases AC2 and AC4 are expressed in hippocampus.

The presence of AC2 and AC4 in hippocampus may account for some of the biochemical and electrophysiological events observed when paired G_s and G_i signals are delivered to hippocampal neurons (Andrade, 1993; Gereau IV and Conn, 1994). Although a role for G_i-mediated potentiation of G_s-stimulated cAMP levels during LTP has not been demonstrated, the fact that pertussis toxin inhibits the development of LTP at several synapses in the hippocampus suggests that G_i-coupled receptor activation plays a role in the events underlying certain forms of LTP (Goh and Pennefather, 1990; Goh and Pennefather, 1989; Ito et al., 1988). Furthermore, since robust increases in cAMP are thought to be required for stimulating cAMP-responsive transcription (Hagiwara et al., 1993), a phenomenon which occurs during LTP in the hippocampus (Impey, 1996), the regulatory properties of AC2 and AC4, as well as their expression in hippocampus, suggests that these adenylyl cyclases play a role in cAMP-dependent synaptic plasticity. Confirmation of these hypotheses will require gene disruption techniques and characterization of hippocampal LTP in the knockout mice.

An additional physiological function for control of circadian melatonin synthesis in the pineal gland (and possibly the retina) has been suggested for AC1. The nighttime induction of the AA-NAT gene in pineal is a well-documented cAMP-dependent process (Baler et al., 1997; Roseboom et al., 1996). AA-NAT induction is synergistically stimulated by α_1- and β_1-adrenergic receptors , indicating that the α_1 (Ca^{2+}/PKC) and β_1 (G_s) components converge upstream of AA-NAT transcription. Interestingly, AC1 is synergistically stimulated by paired G_s and Ca^{2+} signals (Wayman et al., 1994). Along these lines, Tzavara et al demonstrated that the AC1 mRNA undergoes a circadian

oscillation in the rat pineal gland (Tzavara et al., 1996). To begin to define the role of AC1 in pineal function, I have utilized AC1 mutant mice created in this laboratory.

To this point, I have demonstrated that Ca^{2+}/CaM-stimulated adenylyl cyclase activity undergoes a circadian oscillation in mouse pineal, but not in mouse retina. In mouse pineal, Ca^{2+}/CaM-stimulated adenylyl cyclase activity increased ~200% at night relative to the day. In retina, the Ca^{2+}/CaM-stimulated adenylyl cyclase activity was reduced by ~55% compared to wild-type mice. The Ca^{2+} sensitivity of mouse retinal adenylyl cyclase was similar to that for AC1. Additionally, the Ca^{2+} sensitivity of mouse retinal adenylyl cyclase was similar to that for AC1 expressed in HEK 293 cells. These results indicate that the major Ca^{2+}/CaM-stimulated adenylyl cyclase in mouse eye cup preparations in AC1.

To determine whether disruption of the AC1 gene had any effects on a circadian output such as locomotor activity, I monitored the activity of wild-type and mutant mice continuously for 12 days. I found that there are differences in actual activity during periods of relative inactivity (during the light phase). The wild-type mice only have a brief period of inactivity, while the mutant mice are inactive for a greater period of time during the light phase. We refer to this window of time as the "trough" of inactivity. The significance of this observation remains to be established.

A second difference between the activity profiles of wild-type and AC1 knockout mice is in their ability to respond to a shift of the light/dark cycle. Animals were monitored on a 12/12 (L/D) cycle for several days, after which the cycle was switched to a 6/18 (L/D). The wild-type mice seemed to adapt to the phase-shift such that their locomotor activity increased at the new time of dark onset. Interestingly, the locomotor activity of the mutant mice remained entrained to the original 12/12 (L/D) cycle. This result, albeit preliminary, suggests that the AC1 gene disruption may compromise the ability of the mice to adapt to changes in the light/dark cycle. If melatonin regulates the circadian expression of

locomotor activity, the inability of AC1 knockout mice to adapt to phase-shifts may be a result of altered melatonin synthesis in the pineal gland. Future work will be required to elucidate a (possible) connection between AC1, melatonin synthesis, and alterations in AC1 mutant mouse locomotor activity.

Table IV

Regulatory Properties of the Mammalian Adenylyl Cyclases *in vivo* (ca. 1997)

Type	Gs-coupled R	Gi-coupled R	Ca2+	βγ
1	insensitive	inhibited	stimulated	inhibited*
2	stimulated	stimulated	insensitive	stimulated
3	stimulated	ND	inhibited	ND
4	stimulated	stimulated	ND	stimulated
5	stimulated	ND	inhibited	ND
6	stimulated	ND	inhibited	ND
7	stimulated	ND	insensitive	ND
8	insensitive	insensitive	stimulated	insensitive

ND - not determined

* - $\beta\gamma$ inhibits G_S stimulation but does not contribute to G_i-mediated inhibition

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