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**Multiple mechanisms underlying desensitization of the liver  
adenylyl cyclase system: Structure and cAMP regulation of liver  
adenylyl cyclases**

**Premont, Richard Thomas, Ph.D.**

**City University of New York, 1992**

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**MULTIPLE MECHANISMS UNDERLYING DESENSITIZATION  
OF THE LIVER ADENYLYL CYCLASE SYSTEM.  
STRUCTURE AND cAMP REGULATION OF LIVER ADENYLYL CYCLASES.**

by

**RICHARD THOMAS PREMONT**

**A dissertation submitted to the Graduate Faculty in Biomedical Sciences in fulfillment of  
the requirements for the degree of Doctor of Philosophy, City University of New York.**

**1992**

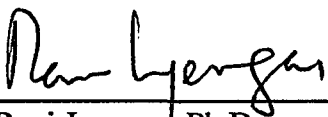
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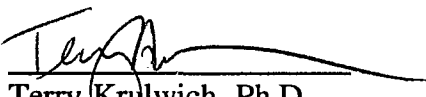
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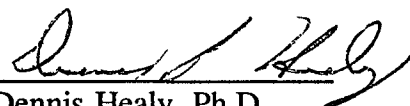
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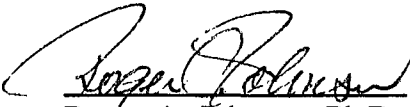
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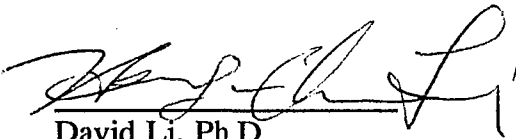
  
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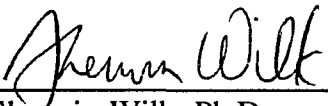
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## Abstract

Multiple Mechanisms Underlying Desensitization of the Liver Adenylyl Cyclase System.

Structure and cAMP Regulation of Liver Adenylyl Cyclases.

by

Richard Thomas Premont

Advisor: Ravi Iyengar

The hormone-sensitive adenylyl cyclase signalling pathway is a multi-component system on the cell surface. Hormone receptors activate the stimulatory guanine nucleotide binding regulatory protein  $G_s$ , which in turn activates the enzyme adenylyl cyclase to convert ATP to cAMP. An important feature of this system is the *desensitization* of response following prolonged or repeated stimulation. Thus, the activity of adenylyl cyclase reflects previous states of activity as well as current stimulation.

I have utilized chick hepatocytes in primary culture to study in detail desensitization of the adenylyl cyclase system in response to glucagon treatment. Homologous desensitization results from regulation of glucagon receptors, which are first uncoupled from  $G_s$  and later removed from the cell surface. Heterologous desensitization involves both loss of  $G_s$  activity and the reduction in the ability of adenylyl cyclase to respond to  $G_s$  stimulation. The reduction in the ability of adenylyl cyclase to respond to  $G_s$  stimulation can be mimicked by treatment of hepatocytes with a cAMP analog, as well as by treatment of membranes with purified protein kinase A and ATP. Since  $G_s$  is not

phosphorylated by protein kinase A, this effect appears due to the direct phosphorylation of adenylyl cyclase by protein kinase A. A similar regulation is also observed in membranes from the mouse S49 lymphoma cell line.

I have further explored the mechanism of regulation of adenylyl cyclase in heterologous desensitization by phosphorylation by the cAMP-dependent protein kinase by deducing the primary structure of the liver form of the enzyme. I have cloned two members of a novel subfamily of adenylyl cyclases from a rat liver cDNA library, and the murine homolog of one of these adenylyl cyclases from the S49 lymphoma (UNC variant) cell line. Both of these adenylyl cyclase isoforms are widely expressed in rat tissues. Both adenylyl cyclases share a consensus site for protein kinase A phosphorylation, which may be responsible for regulation of the enzymes during heterologous desensitization. This new subfamily of adenylyl cyclases, unlike four previously cloned enzymes, appears to have the properties expected of the adenylyl cyclase activity found in most tissues.

## Preface

This thesis is organized according to the *old* guidelines of City University of New York, rather than merely "stapling" together previously published papers and manuscripts. Figures and Tables are interleaved with the text. References for all chapters are located at the end of the text. Chapter 1 provides a general introduction to G protein-regulated signal transduction systems, in particular the hormone-regulated adenylyl cyclase system, and to the phenomenon of desensitization. Chapter 2 describes the methodologies used in these studies. Chapter 3 describes the chick hepatocyte adenylyl cyclase system and the general characteristics of glucagon-induced desensitization in this cell. Regulation of the glucagon receptor in the homologous phase of desensitization is analyzed in detail. This work has been published (Premont & Iyengar, 1988). Chapter 4 concerns analysis of the involvement of G protein regulation in heterologous desensitization in the chick hepatocyte. This work has been published (Premont & Iyengar, 1989). Chapter 5 examines the role of effector-level regulation of the adenylyl cyclase enzyme by cAMP-dependent protein kinase in heterologous desensitization, in both chick hepatocytes and the mouse S49 lymphoma cell line. This work has not yet been published. Chapter 6 analyzes the structure of two distinct adenylyl cyclases from rat liver as determined from the sequence of their cDNAs, and relates this potential structure to regulation of the enzyme by both  $G_s$  and by protein phosphorylation. This work has not yet been published (Premont *et al*, 1992 - submitted), except for the identification of a previously cloned, presumed "calmodulin-insensitive adenylyl cyclase" cDNA sequence as a member of the neural cell adhesion molecule family (Premont, 1991). Chapter 7 summarizes the results

obtained in the preceeding chapters by examining the role of desensitization in the adenylyl cyclase system in light of recent advances in understanding of the components of this signal transduction pathway, particularly the potential role of adenylyl cyclase diversity in differential regulation of this signaling pathway.

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## CHAPTER 1

### INTRODUCTION

Since the pioneering studies of Sutherland and Rall (1958) on the similarity of the actions of glucagon and epinephrine on metabolism in the canine liver led to the discovery of cAMP and its role as a *second messenger*, many intracellular second messenger systems have been identified. Many other second messenger-generating effector systems share common features with the cAMP-generating adenylyl cyclase system, but most significant is the requirement of a guanine nucleotide binding protein to mediate transduction of the signal of an extracellular hormone binding to its receptor protein to the intracellular activation of the effector system. With the continuing unveiling of G protein-mediated signaling mechanisms in sensory transduction and in regulation of homeostasis, growth, and development has come an appreciation of this underlying mechanistic unity and its importance in cellular coordination.

#### The adenylyl cyclase system

##### Glucagon and its physiology

Glucagon is a member of the gastrointestinal (GI) family of polypeptide hormones, which includes such related polypeptides as secretin (the substance for which Starling (1905) coined the term "hormone"), gastric inhibitory peptide (GIP), growth hormone

releasing hormone (GHRH), peptide histidine-isoleucine (PHI), pituitary adenylyl cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP). Glucagon is a 29 amino acid polypeptide hormone which is derived from specific proteolytic processing of proglucagon. Proglucagon can be processed to produce two glucagon-like peptides (GLP-1 and GLP-2), glicentin (which contains the full glucagon sequence), as well as glucagon itself (Bell *et al*, 1983; Lopez *et al*, 1983; Drucker & Asa, 1988; Hasegawa *et al*, 1990). Proglucagon is expressed mainly in the  $\alpha$  cells of the pancreas, but is also found in the L type enteroendocrine cells of the gastrointestinal tract and in certain regions of the brain. Circulating plasma glucagon is mainly secreted by the  $\alpha$  cells of the pancreas, in response to lowered levels of glucose in the blood, although regulation of glucagon release appears to be mediated through both insulin-mediated inhibition of its release and by acute elevation of glucose levels (Unger, 1985).

The major targets of glucagon are the liver and adipose tissue. In both tissues, glucagon alters carbohydrate metabolism in order to assure that an adequate level of glucose is maintained in the blood for the needs of the brain and other tissues. Specifically, glucagon decreases the uptake of blood glucose by these tissues, blocks the conversion of glucose into glycogen, and stimulates the breakdown of stored glycogen into glucose (Farah, 1983). In hepatocytes, glucagon also initiates *de novo* synthesis of glucose and blocks the synthesis of lipids from acetyl CoA. Glucagon produces these metabolic effects by increasing the intracellular concentration of cAMP through activation of the enzyme adenylyl cyclase. Elevation of the intracellular concentration of cAMP increases the activity of the cAMP-dependent protein kinase, or protein kinase A, by binding to and causing the dissociation of the inhibitory regulatory subunits from the

kinase catalytic subunits (Edelman *et al*, 1987). Active protein kinase A phosphorylates many substrate proteins, including phosphorylase kinase, initiating a cascade of specific protein phosphorylation events which leads to the inhibition of the enzymes of glycogen synthesis and the activation of the enzymes of glycogenolysis and gluconeogenesis.

## Receptors

Receptors for hormones and neurotransmitters which cannot penetrate the cell membrane are necessarily located on the surface of the cell. Pharmacologically, receptors have been traditionally defined by the specificity of their binding of agonists and antagonists, and by the specificity of their coupling to an effector system or physiological response (Green, 1990). Various distinct hormone receptors occupy the surface of every cell in multicellular organisms. Target tissues for a particular hormone are defined by the presence of its receptors on the surface of cells in that tissue.

Receptors which activate G protein-coupled effector systems share several common features. These receptors are transmembrane glycoproteins with sizes ranging from 40-100 kDa. The glucagon receptor is a 60 kDa glycoprotein with a protein backbone of 45 kDa as identified by crosslinking of iodinated glucagon (Johnson *et al*, 1981; Iyengar & Herberg, 1984; Padrell *et al*, 1987) or by immunoblotting with anti-receptor monoclonal antibodies (Iwanij & Vincent, 1990). The glucagon receptor has not yet been substantially purified nor has its cDNA been cloned. The recent cloning of canine and human vasoactive intestinal peptide receptors (Libert *et al*, 1989; Sreedharan *et al*, 1991) and a rat secretin receptor (Ishihara *et al*, 1991) should allow cloning of the glucagon

receptor cDNA, as well as those for other members of the GI family of hormones (although the VIP and secretin receptors are members of distinct classes of G protein-coupled receptors which share little sequence similarity, as described below).

Cloning of cDNAs encoding many G protein-coupled receptors has revealed that they fall into at least two (perhaps three) distinct classes, each with some low (20-30%) overall sequence similarity, mainly concentrated in seven hydrophobic domains which have been predicted to be transmembrane spans (Dohlman *et al*, 1987; Strosberg, 1991). There appear to be, however, particular residues within these putative membrane spanning regions which are very highly conserved among cloned members of these superfamilies (see *Table 1-1*). Ligands have been predicted to bind in a pocket within these membrane spans (Dixon *et al*, 1987; Dohlman *et al*, 1988; Matsui *et al*, 1989). In the loops connecting these transmembrane spans, there is greater variability among receptors for different ligands, and among subclasses of receptors which bind a single ligand. Regions responsible for coupling of adrenergic and muscarinic receptors to their G proteins have been localized to the intracellular loop region between the fifth and sixth transmembrane spans (Kobilka *et al*, 1988; O'Dowd *et al*, 1988; Leichleiter *et al*, 1990; Wong *et al*, 1990). Regardless of whether they couple to distinct G proteins (and effectors) or bind quite different types of ligands (small molecules, peptides or large proteins), these receptors appear to share the same overall "bacteriorhodopsin-like" structure.

Agonist binding to receptor can occur with two distinct affinities, high (usually low nM) and low (usually high nM to low  $\mu$ M). Guanine nucleotides promote a shift from the high to low affinity state by increasing the rate of ligand dissociation from the receptor (Rodbell *et al*, 1971c). These affinities are interpreted as reflecting differing

Table 1-1. Conservation near transmembrane domain 7 of cloned G protein-coupled receptors. Single-letter amino acid codes are shown for cloned G-protein-coupled receptors whose ligand has been determined by functional expression. Dashes indicate heterogeneity of amino acids in a given position among members of that subfamily of receptors. Putative receptors whose ligands have not been identified are not shown.

|         |                                                                  |                     |
|---------|------------------------------------------------------------------|---------------------|
| Class 1 | $\beta$ -adrenergic (1,2,3)                                      | W-GY-NS-FNP-IYCRSP  |
|         | $\alpha$ 1-adrenergic (A,B,C)                                    | WLGY-NSC-NPIIYPCSS  |
|         | $\alpha$ 2-adrenergic (C2,C4,C10)                                | W-GYCNSSLNPVIYT-FN  |
|         | serotonergic (1 <sub>A</sub> ,1 <sub>C</sub> ,1 <sub>D</sub> ,2) | W-----S--NP--Y--FN  |
|         | dopaminergic (1,2,3,4,5)                                         | W-G--NS--NP-IY--FN  |
|         | histaminergic (H <sub>1</sub> ,H <sub>2</sub> )                  | WLGY-NS-LNP--Y---N  |
|         | muscarinic (1,2,3,4,5)                                           | WLCY-NST-NP-CYALCN  |
|         | thyrotropin (TRH)                                                | ICIYLNSAINPVIYNLMS  |
|         | adenosine (A <sub>1</sub> , A <sub>2</sub> )                     | -L-H-NS--NP--YA-RI  |
|         | cannabinoid (THC)                                                | MLCLLNSTVNPPIIYALRS |
|         | tachykinin (SP,SK,NMK)                                           | WLAMSSTMYNPPIIYCCLN |
|         | cholecystokinin (CCK <sub>A</sub> )                              | LLSYTSSCVNPPIIYCFMN |
|         | opsins (rhod-,iod-)                                              | -F-KSA-IYNP-IY--MN  |
|         | chemotactic (fMLF-1,2)                                           | -L-F--SCLNP--Y-F-G  |
|         | $\alpha\beta$ -glycoprotein (LH,FSH,TSH)                         | LFYP-NSCANPFLYAIFT  |
|         | somatostatin (1,2)                                               | -L-YANSCANPILY-FLS  |
|         | bradykinin (B <sub>2</sub> )                                     | YVAYSNSCLNPLVYVIVG  |
|         | angiotensin II (1,2)                                             | C-AYFNNCLNPLFYGFLG  |
|         | neuropeptide Y                                                   | ALAFFHCCLNPILYAFLG  |
|         | complement C5a                                                   | SFAYINCCINPPIIYVVAG |
|         | vasoactive intestinal peptide                                    | CLSLVHCCVNPVLYSFIN  |
|         | olfactory (various)                                              | -----TP--NPFIY-LRN  |
|         | thromboxane A <sub>2</sub>                                       | RVATWNQILDWPVYILFR  |
|         | platelet activating factor                                       | CLLSTNCMLDPVIYCFLT  |
|         | thrombin                                                         | CVSSISSCIDPLIYYYAS  |
|         | endothelin (ET <sub>A</sub> , ET <sub>B</sub> )                  | N-A--NSCINPIALY-VS  |
|         | bombesin (GRP,NMB)                                               | -L-F-NSCVNPFALYLLS  |
| Class 2 | secretin                                                         | ALGSFQGLVVAVLYCFLN  |
|         | calcitonin                                                       | SLIHFQGFFVAIIYCFCN  |
|         | parathyroid hormone                                              | LFNSFQGFFVAIIYCFCN  |
| Class 3 | metabotropic glutamate (1-4)                                     | --V-LG----PK--I---  |

conformational states of the receptor, where high affinity requires that the receptor be bound to the appropriate G protein. Isolated receptor binds agonists with only low affinity. Antagonists bind only with lower affinity and are not affected by guanine nucleotides. They are antagonists precisely because, although they bind to the receptor, they cannot promote the conformational change to high affinity state concomitant with activation of the G protein. Ligand activation of the G protein, and hence the effector function, requires the presence of GTP (Rodbell *et al*, 1971a). Receptor-catalyzed activation of the G protein can be achieved in the absence of ligand with GTP and high concentrations of  $Mg^{2+}$ ; the role of agonists is to lower this  $Mg^{2+}$  threshold to below the physiological concentration (Iyengar & Birnbaumer, 1981; 1982). The GTP dependence of the receptor affinity state for agonist binding (but not antagonists) and the GTP requirement for hormone action are the hallmarks of G protein mediation of receptor action.

### G proteins

The dominant feature of the adenylyl cyclase system, and an ever-widening set of other effectors, is the central role of a guanine nucleotide binding regulatory protein (G protein) as mediator between receptors and effector. The absolute requirement for GTP in order to obtain glucagon stimulation of liver adenylyl cyclase (Rodbell *et al*, 1971a) was the first indication of the regulatory role of that nucleotide in signal transduction systems. Enzymatically nonhydrolyzable analogs of GTP, such as GppNHp and GTP $\gamma$ S, extensively and persistently activate adenylyl cyclase (Londos *et al*, 1974; Schramm &

Rodbell, 1975). Hormones abolish the characteristic lag time for GppNHp activation of the liver adenylyl cyclase (Salomon *et al*, 1975) by facilitating both release of bound GDP and binding of the GTP analog (Cassel & Selinger, 1978), as well as stimulate hydrolysis of GTP to GDP and  $P_i$  (Cassel & Selinger, 1976). By reconstitution analysis, Ross and Gilman (1977a,b) found that the missing component in the *cyc<sup>-</sup>* mutant of the S49 murine lymphoma cell line, which lacks hormonal stimulation of adenylyl cyclase, was a guanine nucleotide-binding entity that promoted the coupling between the hormone-receptor complex and the catalyst of adenylyl cyclase. This entity is the G protein now called  $G_s$ , since it mediates stimulation of adenylyl cyclase. The hormone-receptor complex activates adenylyl cyclase by catalyzing the GTP-dependent activation of this guanine nucleotide binding protein (Citri & Schramm, 1980; Iyengar & Birnbaumer, 1982).

The G proteins comprise a family of related  $\alpha\beta\gamma$  heterotrimeric proteins which mediate receptor coupling to various membrane-bound effector systems (Iyengar & Birnbaumer, 1987; Gilman, 1987). The G protein  $\alpha$ -subunit is the unique element of each member of the G protein family, although all  $\alpha$ -subunits are highly similar. The  $\alpha$ -subunits bind and hydrolyze GTP (Fung *et al*, 1981; Northup *et al*, 1983a), and share regions of homology with other known GTP-binding proteins (ras, EF-Tu) (Halliday, 1984). Where it has been tested,  $\alpha$ -subunits with bound GTP (or non-hydrolyzable GTP analogs) are the moieties which stimulate their respective effector functions (Northup *et al*, 1983a; Yatani *et al*, 1989; Imoto *et al*, 1988; Moriarty *et al*, 1990; Taylor *et al*, 1991; Smrcka *et al*, 1991). Some of the  $\alpha$ -subunits also contain sites of modification by ADP-ribosyltransferase bacterial toxins, such as those produced by *Vibrio cholera* and *Bordatella pertussis*. Cholera toxin ADP-ribosylates an arginine residue in the  $\alpha$  subunit

of  $G_i$  and  $G_s$  (Van Dop *et al*, 1984a), leading to greatly reduced GTPase activity of the protein (Cassel & Selinger, 1977). Cholera toxin treatment thus causes the persistent activation of  $G_s$  by making bound GTP "nonhydrolyzable." Pertussis toxin ADP-ribosylates the Cys<sup>63</sup> residue in the  $\alpha$  subunits of the  $G_i$ ,  $G_i$  and  $G_o$  proteins (Manning *et al*, 1984; Hurley *et al*, 1984b; West *et al*, 1985) and prevents the  $\alpha$  subunit from productively interacting with receptor proteins (Van Dop *et al*, 1984b; Murayama & Ui, 1984). Pertussis toxin thus "uncouples" receptors from these G proteins and from activation of their effectors. Some, but not all,  $\alpha$  subunits are post-translationally modified by myristoylation (Buss *et al*, 1987; Jones *et al*, 1990b; Mumby *et al*, 1990).

The  $\beta$  and  $\gamma$  subunits of the G proteins are tightly associated as a dimeric complex under native conditions (Hildebrandt *et al*, 1984b). The  $\beta\gamma$ -complex, by association with the  $\alpha$ -subunit, inhibits the capability of the  $\alpha_s$ -subunit to activate adenylyl cyclase (Northup *et al*, 1983b). The  $\beta\gamma$ -complex is very hydrophobic, and would appear to help anchor the G protein to the plasma membrane (Sternweis, 1986). There are at least 4 forms of the G protein  $\beta$  subunit, of 35-36 kDa (Gao *et al*, 1987; Gao *et al*, 1988; Levine *et al*, 1990). The  $\gamma$  subunits appear to occur in several forms, of 5-10 kDa (Hurley *et al*, 1984a; Gautam *et al*, 1989; Fukada *et al*, 1989; Robishaw *et al*, 1989; Gautam *et al*, 1990). Among purified and resolved G proteins (and presumably within a cell), however, there seems to be no apparent segregation of particular  $\alpha$ -subunits to particular  $\beta\gamma$ -subunits when several are expressed, although there may be some segregation of  $\beta$  and  $\gamma$  combinations (Gautam *et al*, 1990).

G protein transduction of the signal of an agonist binding to its receptor to activation of its coupled intracellular effector system is characterized by both signal

amplification and persistence. Amplification is possible because receptors activate their G proteins in a catalytic manner, in that one agonist-occupied receptor may activate more than one G protein per ligand binding event (Fung & Stryer, 1980; Pedersen & Ross, 1982). Signal persistence occurs through a regulatory cycle of G protein activation and deactivation. Resting or basal state G proteins are  $\alpha\beta\gamma$  heterotrimers with bound GDP (Fung *et al*, 1981; Northup *et al*, 1983a). Agonist-occupied receptor induces in the G protein a conformational change which increases its affinity for  $Mg^{2+}$  and reduces its affinity for guanine nucleotides, allowing release of GDP (Iyengar & Birnbaumer, 1981; 1982). Binding of GTP induces further conformational change leading to the active state of the G protein, capable of activating effector (Codina *et al*, 1984a). The intrinsic GTPase activity of the G protein  $\alpha$  subunit hydrolyzes the bound GTP to GDP and returns the G protein to its inactive conformation (Brandt *et al*, 1983; Brandt & Ross, 1986). Since this GTPase activity is *not* affected by the agonist-occupied receptor, the G protein, once activated, has a characteristic active lifetime which is *independent* of the concentration of agonist. Direct G protein activation by NaF requires GDP, and is thought to be mediated by an  $AlF_4^-$  complex binding to the inactive, GDP-liganded  $\alpha$ -subunit and mimicking the active, GTP-bound state (Bigay *et al*, 1985). Purified GTP $\gamma$ S-liganded  $\alpha$  subunits resolved from  $\beta\gamma$  subunits are capable of persistently activating effectors (Northup *et al*, 1983a; Yatani *et al*, 1987a,b; Moriarty *et al*, 1990; Smrcka *et al*, 1991). Whether the  $\beta\gamma$  subunits actually dissociate from hormonally-activated GTP-bound  $\alpha$  subunits is not clear. Activation of  $G_s$  in liver membranes by glucagon in the presence of GppNHp leads to a reduction in the size of  $G_s$  on sucrose gradients consistent with its being free  $\alpha_s$  subunit (Iyengar *et al*, 1988). Addition of free  $\beta\gamma$  subunits inhibits

the ability of the  $\alpha$  subunit to activate effector (Northup *et al*, 1983b; Moriarty *et al*, 1988; Boyer *et al*, 1989), implying that only the free  $\alpha$  subunit is required to activate an effector. Further, a point mutation of the  $\alpha_s$  subunit which appears to prevent agonist-promoted dissociation from  $\beta\gamma$ -subunits is unable to activate adenylyl cyclase, although the free GTP $\gamma$ S-activated  $\alpha$ -subunit mutant can activate adenylyl cyclase (Lee *et al*, 1992). However, no direct evidence for subunit dissociation following activation by GTP, the natural ligand, has been obtained.

The  $\alpha$ -subunit of  $G_s$  appears as 42 or 52 kDa proteins following SDS-PAGE of purified proteins (Northup *et al*, 1982) or following ADP-ribose labeling catalyzed by cholera toxin using [ $^{32}$ P]NAD $^{+}$  (Cassel & Pfeuffer, 1978). These  $\alpha_s$  forms are distributed distinctly in different tissues: liver and brain contain both 42 and 52 kDa species, while erythrocytes contain only the 42 kDa form. Cloning has revealed that  $\alpha_s$  exists in four distinct forms, two smaller and two larger, which are products of differential RNA splicing of a single gene (Bray *et al*, 1986; Kosaza *et al*, 1988). These four different species all stimulate adenylyl cyclase (Mattera *et al*, 1989a).  $G_s$  has also been reported to mediate stimulation of cardiac calcium channels (Yatani *et al*, 1987b; Imoto *et al*, 1988; Mattera *et al*, 1989a).

Many other G proteins are now known. One of the major problems in the G protein field is the difficulty in assigning purified proteins or cloned sequences as mediators for defined effector systems. Thus the G protein  $G_i$  was defined as the mediator of hormonal inhibition of adenylyl cyclase (Hildebrandt *et al*, 1983; Jakobs *et al*, 1983), but the three isoforms which have been identified by cloning (Jones & Reed, 1987) have been shown to stimulate K $^{+}$  channels (Yatani *et al*, 1987a; 1989) as well as

inhibit adenylyl cyclase activity (Wong *et al*, 1991; Lowndes *et al*, 1991; Hermouet *et al*, 1991). Which adenylyl cyclase isoforms are capable of being inhibited by the  $G_i$ s, however, is not yet known, as will be described in the next section. The G protein  $G_o$  was identified by protein purification (Sternweis & Robishaw, 1984; Neer *et al*, 1984), and has been implicated in inhibition of  $Ca^{2+}$  channels (Hescheler *et al*, 1987), stimulation of  $K^+$  channels (VanDongen *et al*, 1989), and stimulation of a phosphatidyl inositol-specific phospholipase C (Moriarty *et al*, 1990; Padrell *et al*, 1991). The rod and cone transducins or  $G_t$ s mediate light activation of the cGMP phosphodiesterase in the visual system (Fung *et al*, 1981).  $G_{olf}$  (closely related to  $G_s$ ) mediates odorant activation of adenylyl cyclase in the olfactory system (Jones and Reed, 1989; Jones *et al*, 1990a).  $G_q$  and the closely related  $G_{11}$  are recently cloned sequences (Strathmann *et al*, 1989; Strathmann & Simon, 1990) which appear to mediate non-pertussis toxin-sensitive stimulation of phosphatidyl inositol-specific phospholipase C type  $\beta$  (Taylor *et al*, 1990, 1991; Smrcka *et al*, 1991; Wu *et al*, 1992).  $G_z$  is a cloned sequence with an unusual GTPase activity and limited distribution (Fong *et al*, 1988; Matsuoka *et al*, 1988; Casey *et al*, 1990).  $G_z$  has been reported to inhibit adenylyl cyclase activity in a non-pertussis toxin-sensitive manner (Wong *et al*, 1992) and to mediate antigen stimulation of mast cells (Hide *et al*, 1991). In addition, several other putative mammalian G proteins ( $G_{12}$  -  $G_{16}$ ) have recently been identified by PCR cloning techniques (Strathmann *et al*, 1989; Strathmann & Simon, 1990; 1991; Amatruda III *et al*, 1991; Simon *et al*, 1991) for which no functions have yet been identified. Conversely, several effector systems have been identified which display characteristics indicative of G protein regulation, but the particular G protein involved has not yet been identified.

## Adenylyl cyclase

Adenylyl cyclase (often improperly called *adenylate* cyclase) converts ATP to 3':5'-cyclic AMP (cAMP) in the presence of  $Mg^{2+}$  or  $Mn^{2+}$  (Sutherland *et al*, 1962). Most, if not all, tissues contain plasma membrane bound adenylyl cyclase activity which can be stimulated by hormones, through specific receptors coupled to  $G_s$ . Other forms of adenylyl cyclase activity, not activated by hormones or  $G_s$ , are also known to exist in various tissues, such as the soluble enzyme in testis (Braun & Dods, 1975; Neer, 1978a). Only the hormone ( $G_s$ ) regulated adenylyl cyclase will be discussed here. Purified bovine brain membrane adenylyl cyclase reconstituted into phospholipid vesicles with both  $G_s$  and  $\beta$ -adrenergic receptors has been shown to express catecholamine stimulation of adenylyl cyclase activity, indicating that these three components are sufficient for hormonal stimulation of this signal transduction system (May *et al*, 1985; Feder *et al*, 1985). Addition of rhodopsin and  $G_i$  reduces the absolute activity of such a reconstituted system, while increasing the specific stimulation of adenylyl cyclase by hormones (Cerione *et al*, 1986). Other cellular components, however, are certainly required for modulation of the system as seen in intact cells or even isolated membranes, as described below.

The activity of adenylyl cyclase is modulated within the cell by hormone activation of the G protein  $G_s$ , as previously described. The direct binding of  $\alpha_s$  to adenylyl cyclase has been confirmed by the immunoprecipitation of the  $\alpha_s$ -adenylyl cyclase complex by antiserum raised against a synthetic peptide from the C-terminal of  $\alpha_s$ , following activation of the  $G_s$  by treatment with GppNHp or NaF (Simonds *et al*,

1989; Morris *et al*, 1990). Adenylyl cyclase can also be stimulated directly by the diterpine *forskolin* (Seamon *et al*, 1981), even in the absence of  $G_s$  as in *cyc*<sup>-</sup> S49 cell membranes (Seamon & Daly, 1981; Clark *et al*, 1982). However, forskolin stimulation is much higher in the presence of  $G_s$ , and low doses of forskolin which have no direct apparent effect are able to enhance the effect of  $G_s$  stimulation of adenylyl cyclase (Seamon & Daly, 1981b). [<sup>3</sup>H]-Forskolin binds with higher affinity to the activated  $G_s$ -adenylyl cyclase complex than to unactivated adenylyl cyclase in membrane preparations (Seamon *et al*, 1984; Nelson & Seamon, 1986), in intact cells (Barber, 1988), and in solubilized preparations (Nelson & Seamon, 1988). Thus, it appears that forskolin stabilizes the  $G_s$ -adenylyl cyclase interaction, and  $G_s$ , the forskolin-adenylyl cyclase interaction. A site of forskolin binding to the glucose transporter has been localized to one of its 12 transmembrane spans using a radiolabeled photoaffinity analog (Wadzinski *et al*, 1990); its site of interaction with adenylyl cyclase has yet to be defined.

Adenylyl cyclase in neuronal tissues (and to lesser extents in certain others, such as heart, pancreatic islets, and kidney) can also be stimulated by  $Ca^{2+}$ /calmodulin (Brostrom *et al*, 1975; Cheung *et al*, 1975). Early studies of brain adenylyl cyclase indicated that it was composed of a mixture of calmodulin-sensitive and -insensitive forms (Brostrom *et al*, 1977). These activities have been chromatographically separated and purified from rat brain membranes (Coussen *et al*, 1986). Immunoprecipitation and immunoblotting experiments utilizing antibodies which recognize only the CaM-sensitive adenylyl cyclase or which crossreact with non-calmodulin-regulated adenylyl cyclase clearly demonstrate the presence of at least two distinct adenylyl cyclase species in brain and other tissues displaying CaM-sensitive adenylyl cyclase activity (Rosenberg & Storm,

1987; Mollner & Pfeuffer, 1988). Recent work indicates that one molecular form of adenylyl cyclase is stimulated by both  $G_s$  and calmodulin (Smigel, 1986; Harrison *et al*, 1989; Eliot *et al*, 1989; Tang *et al*, 1991).

Adenylyl cyclase activity can also be regulated by inhibitory factors. Hormonal inhibition of adenylyl cyclase is a common effect in many tissues. Such inhibition is blocked by pretreatment with pertussis toxin, and can occur in the absence of  $G_s$  (Hildebrandt *et al*, 1983, Jakobs *et al*, 1983). This inhibition was predicted to be due to the direct action of the G proteins originally identified as " $G_i$ ". However, reconstitution of adenylyl cyclase inhibition using highly purified components has not been successful. Expression of activating (GTPase deficient) mutants of  $\alpha_i$  subunits leads to an inhibition of adenylyl cyclase activity, implying that it is the  $G_i$  proteins which mediate inhibition (Wong *et al*, 1991). Further, the  $\alpha$  subunit of  $G_z$  has also been shown to cause a pertussis toxin *insensitive* inhibition of adenylyl cyclase (Wong *et al*, 1992). Although this result has no known physiological relevance, it is not overly surprising as the  $\alpha_z$  protein has highest similarity to the  $\alpha_i$  family of G protein  $\alpha$  subunits.

Arguments as to whether the  $\alpha$  or  $\beta\gamma$  subunits are the mediators of hormonal inhibition of adenylyl cyclase continue, although the lack of inhibitory effect on adenylyl cyclase of other hormones whose activation of another signal transduction system (*e.g.*, phospholipase C or ion channels) would also liberate free  $\beta\gamma$ -subunits tends to weigh against direct  $\beta\gamma$  effects. The new-found diversity of adenylyl cyclase (see below) raises a more relevant question: *which* adenylyl cyclase isoforms can be inhibited by G proteins? Once an individual adenylyl cyclase is identified which can be inhibited by G proteins, the subunits responsible for this inhibition can be unequivocally established. Surprisingly,

for two cloned adenylyl cyclases, G protein  $\beta\gamma$ -subunits *stimulate* adenylyl cyclase in the presence of activated  $G_s$  (Tang & Gilman, 1991; Gao & Gilman, 1991; Federman *et al*, 1992). This unexpected finding may in part explain the puzzling inability to demonstrate adenylyl cyclase inhibition with purified proteins.

Within the cell, adenylyl cyclase activity may also be modulated by adenosine compounds through the enzyme's "P-site," which is distinct from cell surface purinergic receptors (Johnson *et al*, 1979; Johnson, 1982). Partial purification of adenylyl cyclase (and resolution from  $G_s$ ) using adenosine-agarose chromatography indicates that the P-site is contained on the catalytic unit itself (Johnson, 1982). P-site inhibition of adenylyl cyclase is not competitive with the  $Mg^{2+}$ -ATP substrate, and therefore not due to a direct binding of these compounds to the catalytic site of adenylyl cyclase (Londos & Preston, 1977). Adenosine inhibition of adenylyl cyclase is more pronounced for activated states of the enzyme, since various stimulators (forskolin,  $G_s$  activators,  $Mn^{2+}$ , CaM) lower the  $K_i$  for adenosine or its analogs (Londos & Preston, 1977; Florio & Ross, 1983).

Despite successful solubilization from membranes prepared from mammalian tissues, early attempts to purify adenylyl cyclase were generally unsuccessful due to both the lability of the protein and its low abundance in cellular membranes (*e.g.*, Homcy *et al*, 1978; Neer, 1978b; Bitonti *et al*, 1982; Bender & Neer, 1983). The enzyme remains the least characterized component of the cAMP signal transduction system. Adenylyl cyclase has been purified (mainly from bovine brain) by two distinct affinity chromatography methods, and a great variety of sizes have been claimed for the "pure" enzyme. Whether these various proteins actually represent adenylyl cyclases is not clear, since the specific activities and estimated turnover numbers of the preparations have

varied widely. Allowing such ambiguities, it is not clear whether these preparations contain distinct adenylyl cyclase isoforms, reflect differences between laboratories in size estimation, or are simply misidentified major contaminating proteins. Nevertheless, it has recently become clear that there are in fact *several* adenylyl cyclase isoforms whose regulatory properties may differ significantly.

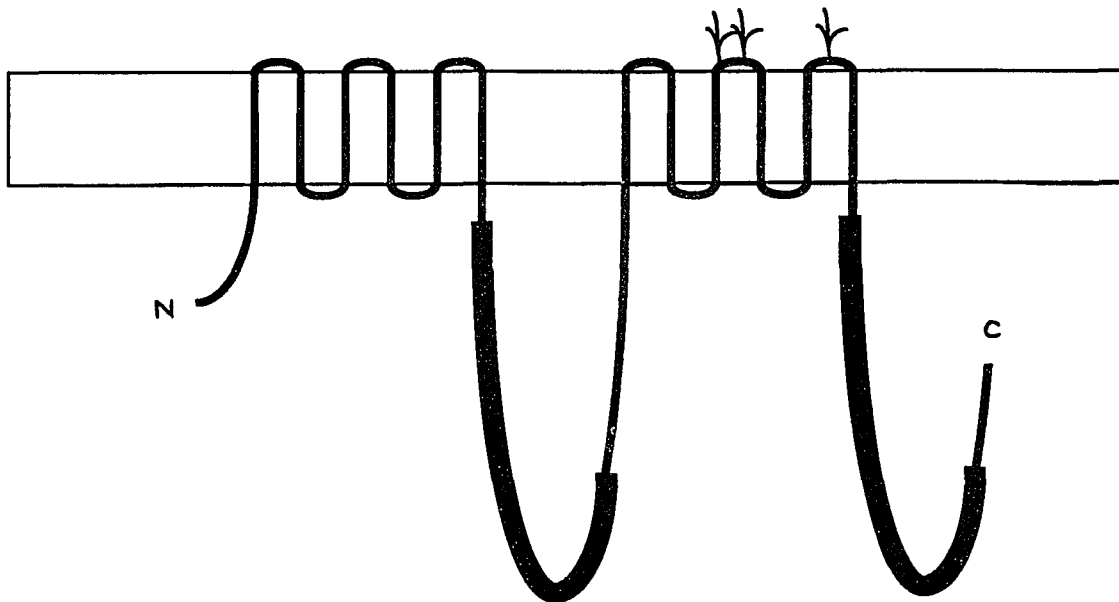
Recognizing the direct activation of adenylyl cyclase by forskolin, Pfeuffer and Metzger (1982) prepared and coupled 7-O-hemisuccinyl-7-deacetylforskolin to aminoethyl-Sepharose as an adenylyl cyclase affinity matrix. Using this affinity support, adenylyl cyclase purified to apparent homogeneity from bovine brain is a 115-120 kDa glycoprotein stimulated by both  $G_s$  and calmodulin (Pfeuffer *et al*, 1985b; Smigel, 1986). Monoclonal antibodies prepared against this purified enzyme also crossreact with calmodulin-insensitive forms of adenylyl cyclase in bovine brain (and other tissues), which were identified as a 150 kDa protein in striatum, and both 150 and 135 kDa proteins in cortex (Mollner & Pfeuffer, 1988). The 150 kDa form has been purified from striatum using forskolin affinity chromatography (Yoshimasa *et al*, 1987). The enzyme was also partially purified from rabbit myocardium, and identified as a 150 kDa glycoprotein (Pfeuffer *et al*, 1985a); this size was later confirmed in immunoblots (Mollner & Pfeuffer, 1988). Adenylyl cyclase from rat olfactory neuroepithelium has been identified as a 185 kDa glycoprotein by immunoprecipitation (Pfeuffer *et al*, 1989). The rat liver enzyme appears to be a 135 kDa protein in immunoblots (Bushfield *et al*, 1990), and the forskolin-purified pig liver enzyme as a 135 kDa glycoprotein which can be crosslinked to [ $\alpha$ - $^{32}$ P]ATP (RTP and RI, unpublished).

The ability of calmodulin to stimulate brain adenylyl cyclase activity has also been used as a tool for the purification of adenylyl cyclase (Wescott *et al*, 1979; Yeager *et al*, 1985; Coussen *et al*, 1985; Zaitseva *et al*, 1988). However, since calmodulin binds to a great number of proteins, there is little specificity for adenylyl cyclase using this affinity chromatography. Brain adenylyl cyclase purified using calmodulin chromatography generally has lower activity than the enzyme purified using forskolin. Following purification on calmodulin or wheat germ lectin matrices, binding of [<sup>125</sup>I]iodo-CaM and [<sup>125</sup>I]iodo-WGL to the purified fractions has been used to "identify" the catalytic unit. Various reports of "pure" calmodulin-purified brain adenylyl cyclases with widely variant turnover numbers and molecular sizes have been published, even within a single laboratory. However, bovine brain adenylyl cyclase purified using both calmodulin and forskolin affinity columns migrates as a single 135 kDa band and retains stimulation by calmodulin following the forskolin affinity purification, indicating that both forskolin and calmodulin can bind to and stimulate the same molecular form of adenylyl cyclase (Minocherhomjee *et al*, 1987; Monneron *et al*, 1987). A polyclonal antiserum raised against this purified protein immunoprecipitates the purified 135 kDa protein, as well as CaM-sensitive adenylyl cyclase activity from brain membrane extracts, and to a lesser extent from certain other tissues (Rosenberg & Storm, 1987).

A cDNA encoding an adenylyl cyclase from bovine brain has recently been cloned (Krupinski *et al*, 1989). A bovine brain cDNA library was screened using oligonucleotides derived from partial amino acid sequences of bovine brain calmodulin-sensitive adenylyl cyclase. The cDNA predicts an 1134 amino acid protein with an internally repeated structure and multiple transmembrane spans. Each repeat contains an

initial hydrophobic region which may contain six transmembrane spans, followed by a large hydrophilic domain predicted to be intracellular (Figure 1-1). This overall structure is comparable (but not homologous) to that of such membrane transporter molecules as the glucose transporter (Mueckler *et al*, 1985; Birnbaum *et al*, 1986), multidrug resistance P-glycoprotein (Gros *et al*, 1986; Chen *et al*, 1986), and cystic fibrosis transmembrane regulator (Riordan *et al*, 1989). Forskolin also has direct effects on other membrane transport and signal transduction proteins, such as the glucose transporter and nicotinic acetylcholine receptor (Laurenza *et al*, 1989). Recent evidence suggests that forskolin binds within the membrane spanning regions of the glucose transporter (Wadzinski *et al*, 1990), and binds the multidrug resistance P-glycoprotein (Morris *et al*, 1991) and perhaps the cystic fibrosis CFTR protein as well. The large, repeated intracellular domains may be involved in nucleotide binding and catalysis, since they both contain some similarities with the catalytic domain of yeast adenylyl cyclases (Kataoka *et al*, 1985; Masson *et al*, 1986; Yamawaki-Kataoka *et al*, 1989) and much more prominent conservation with the catalytic domain of mammalian guanylyl cyclases (Koesling *et al*, 1991). Expression of this cDNA in a baculovirus system yields an activity stimulated by  $G_s$ , forskolin and  $Ca^{2+}$ /calmodulin (Tang *et al*, 1991). Since many tissues contain  $G_s$ -regulated but calmodulin-insensitive adenylyl cyclase, there is at least one more, and in all likelihood, many additional forms of adenylyl cyclase. Indeed, several additional forms of the enzyme, from brain (Parma *et al*, 1991; Feinstein *et al*, 1991), olfactory neuroepithelium (Bakalyar & Reed, 1990), testis (Gao & Gilman, 1991) and liver (*see below*), have also now been cloned by homology screening.

Figure 1-1. Predicted topological structure of mammalian  $G_s$ -stimulated adenylyl cyclases. Comparison of six (and a half) cloned adenylyl cyclase sequences (Shown in Figure 6-8) and prediction of putative membrane-spanning helices in individual sequences indicates a potential structure of two sets of six transmembrane spans separating two large domains. Following Krupinski *et al* (1989), these large domains are predicted to be cytoplasmic, since they contain residues conserved with guanylyl cyclases and to a lesser degree with yeast adenylyl cyclases. Therefore, both the N- and C-terminii are predicted to be inside the cell. Each cytoplasmic domain contains a large region which is highly conserved among the mammalian adenylyl cyclase sequences cloned to date, and are predicted to be the site of the catalytic activity (darkened bars).



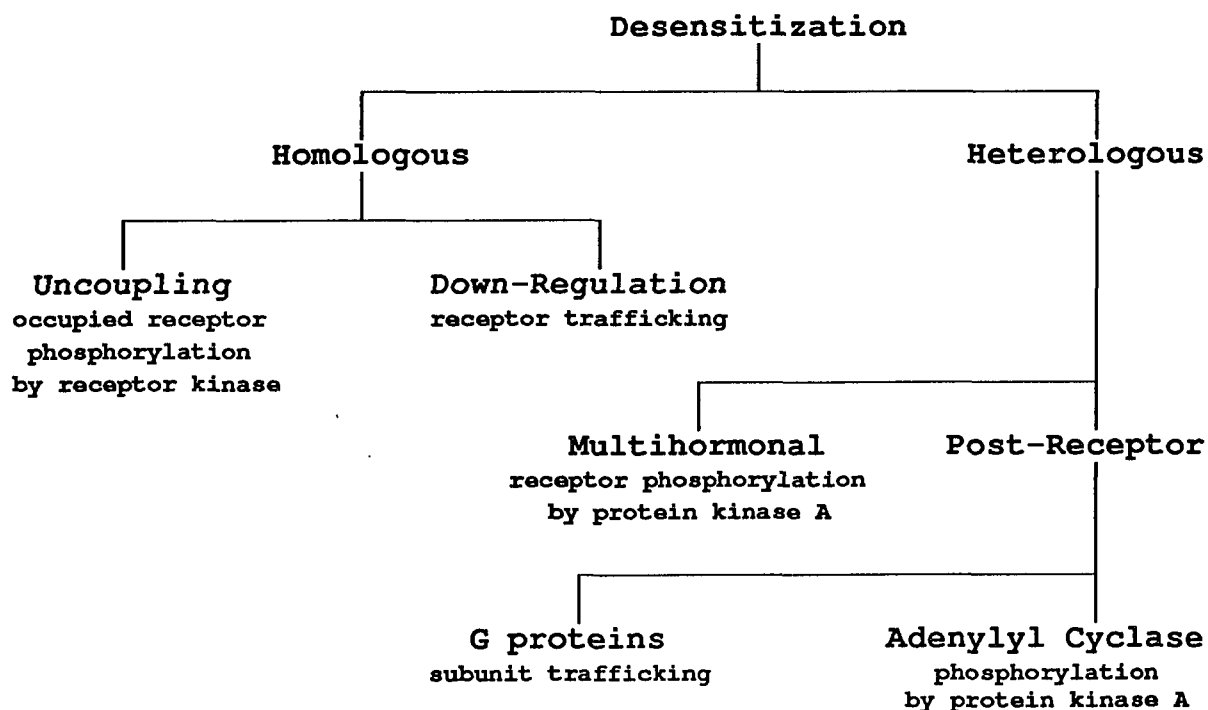
## Desensitization

A common feature of systems regulated by hormones is that their responsiveness wanes upon prolonged or repeated exposure to the hormone. This decreased responsiveness is called *tachyphylaxis* or *desensitization*. Functional desensitization within the intact cell can involve an increased rate of degradation of second messenger molecules as well as a decreased rate of synthesis (Clark, 1986). Further, regulation by hormones of the rates of synthesis and turnover of protein components (Doss *et al*, 1981; 1985) or of the mRNA encoding these components (Collins *et al*, 1989) of the signal transduction system are also potential long-term mechanisms for desensitization. However, for the work described here, only the short-term, membrane-delimited steps in second messenger synthesis were considered.

Desensitization responses can be functionally divided into two broad classes: *homologous* and *heterologous* (Su *et al*, 1980). Homologous desensitization describes a hormone-specific loss of responsiveness, seen only for the hormone used to induce the desensitization (or for other drugs using the same receptor). Heterologous desensitization refers to nonspecific loss of responsiveness, in which the response to hormones in addition to that used to induce desensitization, or even to post-receptor activators, is also reduced. A scheme for classification of the various possible types of homologous and heterologous desensitization and their proposed mechanisms is shown in *Figure 1-2*; evidence for these mechanisms is presented in the text.

Among cellular adenylyl cyclase systems, the most detailed studies delineating the mechanisms involved in the desensitization process have been carried out using the

**Figure 1-2.** Schematic representation of the pathways for desensitization of the adenylyl cyclase system. This scheme presents several distinct pathways by which desensitization of the hormone-stimulated adenylyl cyclase system may occur in various tissues and cell types. Proposed mechanisms for each pathway are discussed in the text.



$\beta$ -adrenergic receptor-stimulated adenylyl cyclase systems of S49 murine lymphoma cells and turkey erythrocytes by Lefkowitz and co-workers, and of 1321N1 human astrocytoma cells by Harden, Perkins and co-workers (*see reviews by* Harden, 1983; Sibley & Lefkowitz, 1985; Clark, 1986). Since numerous hormones and neurotransmitters have their effects by modulating the activity of adenylyl cyclase, it is of interest to know if the mechanisms involved in the development of tolerance in the  $\beta$ -adrenergic receptor system are more generally valid. In addition, desensitization by  $\beta$ -adrenergic agonists is generally homologous in most cells studied, although multihormonal responsiveness can also be affected in some systems. Thus heterologous desensitization, and particularly non-receptor modes of heterologous desensitization, has been much less intensively studied than homologous desensitization.

#### Homologous desensitization

Homologous or hormone-specific desensitization involves, almost teleologically, modulations of the receptors for the desensitizing hormone, and appears to result from two temporally distinct events: uncoupling of the activated hormone receptors from  $G_s$ , followed receptor sequestration from  $G_s$  and down-regulation from the cell surface. Upon agonist occupancy, the  $\beta$ -adrenergic receptor has been observed to undergo a rapid functional uncoupling from  $G_s$ , as determined by loss of high affinity binding sites for agonists and loss of stimulation of adenylyl cyclase, without loss of low affinity, antagonist binding sites (Harden *et al*, 1979). Upon prolonged exposure, receptors are lost from the cell surface by an internalization process (Su *et al*, 1980). Once

internalized, the receptors are either stored for return to the cell surface or are degraded within the cell (Doss *et al*, 1981).

Receptor uncoupling from  $G_s$  is the initial response in homologous desensitization. In the  $\beta$ -adrenergic system, uncoupling is characterized by loss of stimulation of adenylyl cyclase along with loss of high affinity agonist displacement of antagonist receptor probes (Su *et al*, 1980). This uncoupling has been shown to be related to phosphorylation of the receptor by the  $\beta$ -adrenergic receptor kinase (called BARK), an agonist-occupied-receptor-specific protein kinase (Benovic *et al*, 1986b, Benovic *et al*, 1989). The role of BARK phosphorylation of the  $\beta$ -adrenergic receptor in homologous desensitization is thought to be similar to that of rhodopsin kinase in the visual system. Rhodopsin kinase phosphorylates the light-activated form of rhodopsin (equivalent to a hormone-occupied receptor). This phosphorylated rhodopsin is then bound by a protein variously called retinal 48 kDa protein, S-antigen, or arrestin. Arrestin binds to phosphorylated but not unphosphorylated rhodopsin, and prevents rhodopsin binding and activation of transducin, the retinal G protein (Wilden *et al*, 1986). Arrestin thus "uncouples" activation of the light receptor from activation of its G protein in a manner equivalent to homologous desensitization to hormones. In fact, rhodopsin kinase can weakly phosphorylate agonist-occupied  $\beta$ -adrenergic receptors, and BARK can weakly phosphorylate light-bleached rhodopsin (Benovic *et al*, 1986a). Two distinct forms of BARK and one rhodopsin kinase have been identified by cDNA cloning (Benovic *et al*, 1989; Benovic *et al*, 1991; Lorenz *et al*, 1991). A "muscarinic receptor kinase" activity which phosphorylates agonist-occupied muscarinic receptors and light-bleached rhodopsin has

also been purified from bovine brain (Haga & Haga, 1989; 1990; 1992), but its relationship to the BARKs has not yet been determined.

BARK-phosphorylated  $\beta$ -adrenergic receptors couple almost normally to  $G_s$  in reconstituted systems, but appear somewhat more uncoupled when retinal arrestin is added (Benovic *et al*, 1987). Therefore, it was predicted that a somatic cell equivalent of retinal arrestin physically uncouples the BARK-phosphorylated hormone receptors from  $G_s$  in homologous desensitization. Such an arrestin-analog ( $\beta$ -arrestin) has recently been identified by cDNA cloning, and the expressed protein has been shown to increase uncoupling of BARK-phosphorylated  $\beta$ -adrenergic receptors from  $G_s$  (Lohse *et al*, 1990).

Removal of receptors from the plasma membrane constitutes the second phase of homologous desensitization. Studies using antagonist probes for  $\beta$ -adrenergic receptors indicate that following uncoupling from  $G_s$ , receptors are first "sequestered" and then down-regulated. Receptors are said to be sequestered when the binding sites become inaccessible to membrane-impermeant probes but remain available for rapid return to the cell surface (Su *et al*, 1979). Sequestered receptors are found associated with small vesicular fractions of lysed cells (Waldo *et al*, 1983), which appear to be associated with the plasma membrane, since they can be recoupled to adenylyl cyclase following PEG-induced membrane fusion of desensitized cells (Strasser & Lefkowitz, 1985). Down-regulation includes internalization of receptors to some less-accessible intracellular store which leads to eventual receptor degradation (Su *et al*, 1980). Receptors recover rapidly from sequestration, but down-regulated receptors may require prolonged periods and protein synthesis for recovery (Doss *et al*, 1981). Studies with expressed mutant  $\beta$ -adrenergic receptors lacking putative BARK phosphorylation sites indicate that this

phosphorylation is not required for sequestration (Strader *et al*, 1987). However, the ability of the receptor to couple to  $G_s$  (but not necessarily to actual cAMP production) is required for sequestration and down-regulation (Cheung *et al*, 1989; 1990; Rich & Iyengar, 1989).

### Heterologous desensitization

Heterologous desensitization may result from alterations at any level of the signal transduction process: the receptors, G proteins, or effector. Studies of heterologous desensitization in  $\beta$ -adrenergic systems, particularly in avian erythrocytes and S49 lymphoma cells, have mainly focused on receptor regulation. Other levels of regulation have been studied mainly in phenomenological terms, and evidence for the involvement of particular mechanisms are generally lacking.

The best studied form of heterologous desensitization is the 'multihormonal' form, where stimulation by hormones in addition to that used to induce desensitization, but not post-receptor activators, is reduced. Since NaF and guanine nucleotide activation of adenylyl cyclase is not reduced, the defect in the desensitized cells must reside with the receptors or their coupling to  $G_s$ . In both avian erythrocytes and S49 cells, this type of effect is cAMP-mediated and associated with phosphorylation of  $\beta$ -adrenergic (and presumably other) receptors (Sibley *et al*, 1984b; Clark *et al*, 1988). Purified  $\beta$ -adrenergic receptor can be phosphorylated by protein kinase A (Benovic *et al*, 1985). Mutant  $\beta$ -adrenergic receptors lacking one of two protein kinase A phosphorylation sites have been shown to no longer undergo heterologous desensitization when expressed in

mouse L cells (Clark *et al*, 1989). Purified, desensitized (ostensibly protein kinase A-phosphorylated)  $\beta$ -adrenergic receptors have been reconstituted with  $G_s$  in phospholipid vesicles and found to have impaired coupling to  $G_s$  by one group (Strulovici *et al*, 1984) or to have no impairment of coupling by another (Keenan *et al*, 1986). As with BARK phosphorylation, other cellular factors may be important in physically uncoupling the phosphorylated receptors from  $G_s$ .

In many cell systems, particularly for the peptide hormones, heterologous desensitization of adenylyl cyclase is associated with decreased responsiveness to post-receptor activators of the adenylyl cyclase system, such as NaF and guanine nucleotide analogs, as well as to hormones (Clark, 1986; Reithmann *et al*, 1991). This loss of NaF stimulation of adenylyl cyclase has been reported for glucagon-induced desensitization of MDCK cells (Rich *et al*, 1984) and rat Sertoli cells (Attramadal *et al*, 1988). Rat hepatocytes exhibited this effect for both glucagon- (Noda *et al*, 1984) and  $PGE_1$ -induced desensitization (Garrity *et al*, 1983). This desensitization is generally associated with a loss of  $G_s$  activity as assayed by reconstitution in *cyc*<sup>-</sup> S49 membranes. The mechanisms most often proposed are loss of  $G_s$  activity due to either covalent modification of  $G_s$  or due to reduction of  $G_s$  levels in the plasma membrane. Many G proteins seem particularly resistant to phosphorylation by known second messenger-regulated protein kinases (*e.g.*, Premont & Iyengar, 1989; Carlson *et al*, 1989; Lounsbury *et al*, 1991), although such modification has been reported for some G proteins in some systems (Katada *et al*, 1985; Zick *et al*, 1986; Carlson *et al*, 1989; Gundersen & Devreotes, 1990; Lounsbury *et al*, 1991). G proteins have been reported to undergo

redistribution within the cellular membrane system in response to agonists (Crouch *et al*, 1989; Haraguchi & Rodbell, 1990; Reithmann *et al*, 1991).

Finally, modulation of the effector itself may also play a role in heterologous desensitization. Such regulation would add a further level of control and interplay between signaling pathways. No direct evidence for the *physiological* regulation of adenylyl cyclase in heterologous desensitization has been published. There are, however, many reports of regulation of adenylyl cyclase in the phenomena of *sensitization*. The functional opposite of desensitization, sensitization is the enhancement of the signaling process following exposure of the system to some drug. Many studies have shown that treatment of cells with active phorbol esters induces an increase in adenylyl cyclase activity (*eg.*, Bell *et al*, 1985). Such sensitization of adenylyl cyclase is thought to be related to phorbol ester activation of protein kinase C and phosphorylation of some components of the adenylyl cyclase system. In frog erythrocytes, this TPA-induced increase in adenylyl cyclase stimulation is quite dramatic (Sibley *et al*, 1986a). Yoshimasa *et al* (1987) demonstrated the TPA-induced phosphorylation of adenylyl cyclase in  $^{32}\text{PO}_4$ -labeled frog erythrocytes by purifying the enzyme on deacetylforskolin-agarose columns and eluting enzyme activity with forskolin, but not inactive forskolin analogs. They also purified a 160 kDa adenylyl cyclase from bovine striatum on forskolin-agarose and demonstrated its *in vitro* phosphorylation by protein kinase C. The size of their bovine brain protein (160 kDa) is not in agreement with the 120 kDa molecule purified from bovine cortex (Pfeuffer *et al*, 1985b; Smigel, 1986), but is consistent with immunoblots showing a predominant 150 kDa CaM-insensitive adenylyl cyclase but little 120 kDa CaM-sensitive adenylyl cyclase in bovine striatum (Mollner &

Pfeuffer, 1988). Further, phorbol ester-induced phosphorylation of a 150 kDa adenylyl cyclase protein has been directly demonstrated in human platelets by immunoprecipitation (Simmoteit *et al*, 1991).

In an unreviewed book chapter, Yoshimasa *et al* (1988) reported the phosphorylation of the purified 160 kDa bovine brain adenylyl cyclase by both protein kinase A and protein kinase C to 1 mol phosphate per mol protein. When reconstituted with  $G_s$  in phospholipid vesicles, this purified adenylyl cyclase preparation phosphorylated by protein kinase C exhibited increased activity, and phosphorylated by protein kinase A exhibited *reduced* activity when compared with unphosphorylated enzyme. The enzyme in detergent solution exhibited no change in activity regardless of phosphorylation state. Recently, a purified preparation of sea urchin sperm CaM-sensitive adenylyl cyclase has been shown to be an *in vitro* substrate for protein kinase A, although no effects were seen on activity (Bookbinder *et al*, 1991). The recently cloned calmodulin-sensitive adenylyl cyclase cDNA from bovine brain contains one consensus site each for protein kinase A and protein kinase C (Krupinski *et al*, 1989). The rat olfactory (type 3) and rat brain (type 2) enzymes have a single putative site each for protein kinase A and several for protein kinase C (Bakalyar & Reed, 1990; Feinstein *et al*, 1991). The rat testis (type 4) enzyme has no predicted protein kinase A sites but several sites for protein kinase C (Gao & Gilman, 1991). None of the protein kinase A consensus sites are in conserved locations among these four forms of adenylyl cyclase, and the predicted protein kinase C sites are scattered throughout the sequences. Thus the distinct adenylyl cyclases may be differentially regulated by protein kinases, as will be further discussed in Chapters 6 and 7.

## **General and specific aims of this thesis**

The studies described here were undertaken in order to develop an understanding of the molecular mechanisms underlying the various aspects of hormonal desensitization of the adenylyl cyclase system. Chick hepatocytes in primary culture were used as a model for the liver adenylyl cyclase system. Chick hepatocytes are relatively easily maintained in a non-proliferating culture for several days, compared with the more widely used, but more difficult, rat hepatocyte primary culture system. My initial aim was to characterize the suitability of cultured chick hepatocytes for studies of the regulation of the adenylyl cyclase system. The cells were found to maintain an intact adenylyl cyclase system which can be stimulated by a variety of hormones, and to exhibit a robust multicomponent desensitization response.

### **1. Characterization of the desensitization response:**

Adenylyl cyclase activity measurements, glucagon receptor binding assays, and specific immunoblotting for G protein subunits were used to quantify alterations in the hormone-stimulated adenylyl cyclase system following desensitization. Evidence was found for two temporally and functionally distinct phases of glucagon receptor regulation (rapid receptor uncoupling from  $G_s$ ; slower receptor internalization). Additional loss of plasma membrane  $G_s$  activity and cAMP-induced reduction in activity of the adenylyl cyclase enzyme was also observed.

### **2. Regulation of adenylyl cyclase by protein kinase A.**

Of the various desensitization components observed in the hepatocyte studies, the apparently direct regulation of the activity of adenylyl cyclase by cAMP had not been previously reported. I therefore investigated the potential for negative-feedback regulation

of adenylyl cyclase activity through protein kinase A-mediated phosphorylation. In both chick hepatocytes and murine S49 lymphoma cells, cAMP treatment of cells and protein kinase A (plus ATP) treatment of membranes promotes similar reduction in the activity of adenylyl cyclase.

### 3. Identification of adenylyl cyclase isoforms from the liver.

The cloning of a brain (calmodulin-stimulated) adenylyl cyclase made it possible to undertake studies aimed at identifying just how protein kinase A (or other) phosphorylation of adenylyl cyclase may regulate the enzyme. Two distinct adenylyl cyclase sequences were cloned from rat liver cDNA using a probe from the brain enzyme. These two enzymes (types 5 and 6) are distinct from four previously cloned mammalian adenylyl cyclases and form a novel subfamily of adenylyl cyclases. Both enzymes appear to be very widely distributed in rat tissues, and share a predicted protein kinase A phosphorylation site in a conserved domain near transmembrane span 7. This site is not shared with any other adenylyl cyclase form currently known, but is conserved in the mouse homolog of type 5 adenylyl cyclase cloned from the murine S49 lymphoma cell line. Expression of these cloned enzymes will allow the direct determination of the effects of protein kinase phosphorylation on the activity of individual adenylyl cyclases.

## CHAPTER 2

### METHODS & EXPERIMENTAL PROCEDURES

#### Chemicals and Reagents

Cesium chloride was from Accurate Chemicals. Ammonium persulfate, bis-acrylamide, sodium dodecyl sulfate and TEMED were from Bio-Rad. 3'-AMP, AppNHp, cAMP, GppNHp, GTP $\gamma$ S, HEPES, NAD,  $\beta$ -NADH, and NMN were from Boehringer Mannheim. Crystalline bovine glucagon and insulin were from Elanco (Lilly). NuSieve GTG and SeaKem agaroses were from FMC Bioproducts. Recrystallized acrylamide was from Fluka. Bluo-gal, BME vitamins, chicken serum, DMEM media powder, Earle's salts, deionized formamide, horse serum, IPTG, kanamycin sulfate, redistilled phenol, and trypan blue were from Gibco/BRL. Recrystallized urea was from ICN. Sodium fluoride was from Malinkrockt. Nitrocellulose circles (85 mm) were from Millipore. Nuflow cellulose acetate filters (25 mm) were from Oxoid. WIPTIDE (Walsh heat-stable protein kinase A inhibitor protein, synthetic 5-22 amide) was from Peninsula. Bovine testis calmodulin was from Pharmacia/LKB. EDAC, HSAB, IODOGEN and MBS were from Pierce. Agarose, amino acids, ampicillin, angiotensin II, ATP (grade I), 8-bromo-cAMP, calcitonin gene-related peptide, creatine phosphate, cyanogen bromide, cycloheximide, diethyl pyrocarbonate, ethidium bromide, forskolin, guanidinium thiocyanate, leupeptin, prostaglandin E<sub>1</sub>, soybean trypsin inhibitor, tetracycline, Tween 60, vasoactive intestinal peptide and 8-arginine vasopressin were from Sigma. Cholic acid from Sigma was purified by recrystallization from ethanol 5 times; 10% solutions were

adjusted to pH 7.5 and deionized by passage through Chelex AG-501. Lubrol-PX was obtained from Sigma, and a 10% (w/v) solution was deionized by passage through Chelex AG-501 prior to use, or was obtained from Pierce (Surfact-Amps 10%). Nitrocellulose sheets (BA 85) and 150 mm circles and Nytran Nylon membranes were from Schleicher & Schuell. Other reagents were of the finest grade available.

### Enzymes

Glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, and 3-phosphoglycerate kinase were from Boehringer Mannheim. Collagenase (type CLS III) was obtained from Cooper Biomedical. Pertussis toxin was from List Biologics. Klenow fragment of DNA polymerase I, restriction enzymes, Taq DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase were from Promega or New England Biolabs through the Brookdale Center for Molecular Biology "store". Cholera toxin, creatine phosphokinase, DNase I, endoglycosidase F, glycopeptidase F, lysozyme, myokinase, and RNase A were from Sigma. RNase-free DNase and phage T3 and T7 RNA polymerases were from Stratagene. Modified T7 DNA polymerase (Sequenase) and Taq polymerase (TAQuence) were from US Biochemical. The catalytic subunit of protein kinase A purified from porcine heart by the method of Flockhart and Corbin (1984) was the gift of Dr. J. D. Corbin (Vanderbilt University, Nashville TN), or was purchased through Promega.

### Radiochemicals

[<sup>3</sup>H]-L-leucine (130 Ci/mmol) and [<sup>35</sup>S]methionine (1200 Ci/mmol) were from

Dupont/NEN. All other isotopes were purchased from ICN Radiochemicals. [ $\alpha$ - $^{32}\text{P}$ ]ATP (50 Ci/mmol) and [ $\gamma$ - $^{32}\text{P}$ ]ATP (6000 Ci/mmol) were synthesized from [ $^{32}\text{P}$ ]H<sub>3</sub>PO<sub>4</sub> (carrier free) using the procedure of Walseth and Johnson (1979), and [ $^{32}\text{P}$ ]NAD<sup>+</sup> (5000 Ci/mmol) by the procedure of Cassel and Pfeuffer (1978) by Dr. Iyengar. [Tyr<sup>10-125</sup>I]moniodoglucagon ([ $^{125}\text{I}$ ]MIG) (2200 Ci/mmol) was prepared by reaction with carrier free Na<sup>125</sup>I and IODOGEN, and purified by reverse-phase HPLC on a C<sub>18</sub> column by the method of Rojas *et al* (1983). Affinity-purified goat antibodies against IgG or protein A were iodinated using Na[ $^{125}\text{I}$ ] and Chloramine T as described by Iyengar *et al* (1987). Purified protein preparations were iodinated using Na[ $^{125}\text{I}$ ] according to Greenwood *et al*, 1963. Oligonucleotides were labeled by phosphorylation with [ $\gamma$ - $^{32}\text{P}$ ]ATP (7000 Ci/mmol) and phage T4 polynucleotide kinase (Sambrook *et al*, 1989). Double stranded DNA probes were prepared by incorporation of [ $\alpha$ - $^{32}\text{P}$ ]dCTP (3000 Ci/mmol) using Klenow fragment of DNA polymerase 1 primed from random hexamers, using the Promega prime-a-gene kit according to the manufacturer's protocol.

### Antisera

Affinity purified goat anti-rabbit IgG (native and conjugated to horseradish peroxidase) and goat anti-rat IgG antisera were obtained from Calbiochem. Purified rat monoclonal antibodies against neonatal mouse brain N-CAM (Hirn *et al*, 1981) were obtained from Chemicon International. Antiserum RM/1 (Simonds *et al*, 1989) was purchased from NEN/Dupont. Recombinant protein A was from Sigma.

Antisera A-584 and U-49 were the kind gift of Drs. Susanne Mumby and Alfred G. Gilman (University of Texas Health Science Center, Dallas TX). Antiserum A-584

was prepared against a synthetic peptide (CKQLQRDRQVYRATHR) common to all four  $G_s$   $\alpha$ -subunits as described for antiserum A-572 (Mumby *et al*, 1986). A-584 also recognizes the  $\alpha$ -subunit of  $G_{olf}$ , but not other G protein  $\alpha$ -subunits. Antiserum U-49 was prepared against the synthetic peptide CEGNVRVSRELAGHTGY from the 36 kDa  $\beta_1$ -subunit of G proteins (Mumby *et al*, 1986), and does not cross-react with the 35 kDa  $\beta_2$ -subunit isoform (Gao *et al*, 1988). Cross reactivity with the  $\beta_3$ -subunit has not been tested.

Antiserum AS/6 was the kind gift of Dr. Allen Spiegel (National Institutes of Health, Bethesda MD). AS/6 was raised against a synthetic peptide (KENLKDCGLF) derived from the carboxy terminal of  $\alpha_i$ , and crossreacts with the three  $\alpha_i$  proteins (Falloon *et al*, 1986) as well as a 43 kDa G protein  $\alpha$ -subunit ( $\alpha_{43}$ ) in human erythrocytes which appears related to  $\alpha_{i3}$  (Carty and Iyengar, 1990).

Monoclonal antibody BBC-4 was the gift of Dr. Thomas Pfeuffer (University of Würzburg, Federal Republic of Germany). This monoclonal antibody was raised against purified bovine brain calmodulin-stimulated adenylyl cyclase, and is capable of recognizing and immunoprecipitating calmodulin-insensitive adenylyl cyclases from various tissues and species in the *denatured* state (Mollner & Pfeuffer, 1988; Mollner *et al*, 1991; Simmoteit *et al*, 1991).

Antiserum S1 was raised in rabbits against the  $\alpha_s$  carboxyl terminal decapeptide sequence (RMHLRQYELL $\text{C}$ ), and is essentially equivalent to antiserum RM/1 of Simonds *et al* (1989). Antiserum S1 recognizes recombinant  $\alpha_s$  but not other expressed  $\alpha$  subunits. Antiserum AC-1 was raised against a synthetic peptide (IGARKPQYDIWGNT $\text{C}$ ) from the second intracellular domain of adenylyl cyclase and common to all currently known

mammalian adenylyl cyclase sequences. Antisera AC-A1 and AC-B1 were prepared against synthetic peptides from sequences unique to the rat type 6 (CGGNQVSKEMKR-MGFEDPKD) and type 5 (CSRTKDSKAFRQMGIDDSSK) adenylyl cyclases, respectively.

Peptides were synthesized manually by Dr. Angeliki Bükü, Department of Physiology and Biophysics, and the purified peptides were conjugated to keyhole limpet hemocyanin through the added cysteine residue using MBS as described in Premont *et al* (1989). Conjugate (300 µg in 1.5 mL) was mixed 1:1 with Freund's adjuvant, and injected intradermally in small pockets on the backs of rabbits. Initial injections used complete adjuvant and all boosts were with incomplete adjuvant. Rabbits were boosted at 5 week intervals and bled on days 7 and 14 after immunization, following the immunization schedule of Green *et al* (1982). Serum was obtained from overnight clots by centrifugation at 1000xg, and stored in aliquots at -70°C.

#### Affinity media

Affi-Gel 102 was from Bio-Rad, and Ultrogel AcA-34 was from IBF. Mono Q HR 5/5 columns and an FPLC system were from Pharmacia/LKB. Reacti-gel 6X was from Pierce. DEAE-Sephacel and Sepharose CL-4B were from Sigma.

7-succinyl-7-deacetylforskolin was synthesized following the protocol of Smigel (1986). Forskolin (250 mg) was dissolved in 2 ml of methanol, and 500 µl of 5 N KOH added for 90 min at 30°C. Glacial acetic acid (~150 µl) was added to reduce the pH to 6.0 and the mixture dried by rotary evaporation. The reaction mixture was extracted five times with 2 ml diethyl ether, and the pooled ether dried under a stream of N<sub>2</sub> to yield

7-deacetylforskolin. This product was dissolved in 2 ml anhydrous pyridine, added to 450 mg succinic anhydride in a dried flask, and mixed for 20 hours at 55°C. The reaction was stopped by addition of water (1 ml), and continued mixing for 2 hours at 40°C. The mixture was extracted twice with 2 ml benzene, and the pooled benzene extracted four times with 3 ml 0.6 N HCl. The extracted benzene was dried under N<sub>2</sub> to yield a brown oil. This succinylation reaction product was purified on a 20cc column of silica gel developed in benzene:ethyl acetate (1:1), and visualized by spotting fractions on silica TLC plates and developing with concentrated sulfuric acid mist following Pfeuffer *et al* (1985a). In this system, forskolin gave an R<sub>f</sub> value of 0.65 and 7-deacetylforskolin an R<sub>f</sub> value of 0.55. Fractions containing 7-succinyl-7-deacetylforskolin (R<sub>f</sub> 0.40) were pooled, and the ability of the product peak to stimulate adenylyl cyclase was verified in S49 cell membranes. Coupling to Affi-Gel 102 amino agarose matrix was according to the protocol of Smigel (1986). 20 ml of Affi-Gel 102 was washed stepwise into 75% N,N-dimethylformamide and 109 mg of purified 7-succinyl-7-deacetylforskolin (233 µmol) was added. The pH was adjusted to 4.7 with 100 mM HCl, and 240 mg EDAC added to the gel slurry while mixing. The pH was maintained at 4.7 by addition of HCl for 1 hour and the reaction allowed to continue for 16 hours. The slurry was washed stepwise into water and washed with 1 M NaCl before use.

Anti-adenylyl cyclase monoclonal antibody BBC-4 (300 µl lyophilized ascites) was rehydrated in 3 ml of 100 mM borate (pH 8.5), 0.9% NaCl, and mixed for 48 h with borate buffer-washed Reacti-gel 6X (1 cc). The gel was incubated a further 2 h with 2 M Tris (pH 8.0) to block remaining sites, washed with PBS, and stored with 0.02% NaN<sub>3</sub> at 4°C.

### Oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer by the DNA Core Facility of the Brookdale Center for Molecular Biology or by Ofer Jacobowitz or Jianghao Chen of this laboratory. When required (for PCR primers), oligonucleotides were purified by separation on 10% polyacrylamide gels (acryl:bis 40:2) containing 7 M urea in TBE buffer at 500 V. DNA was visualized by UV shadowing onto fluorescent TLC plates, and the full-length band excised with a clean scalpel. The minced gel slice was incubated for 16 hours at room temperature with shaking in 5 mL 0.5 M ammonium acetate (pH 5.2), 5 mM EDTA. The eluate was diluted 10-fold with water, and bound to a 0.5cc column of DE-52 DEAE-cellulose (Whatman). The column was washed with 5 mL of 50 mM TEAB buffer and eluted in 1 mL of 1 M TEAB. 1 M TEAB buffer is prepared from 1 M triethylamine through which CO<sub>2</sub> has been bubbled to reduce the pH to 8.6. The eluate was evaporated to dryness twice and resuspended at 100 µM based on absorbance at 260 nm.

### Cell culture solutions and media

Phosphate buffered saline (PBS), containing (g/l): KCl (0.2), KH<sub>2</sub>PO<sub>4</sub> (0.2), NaCl (8.0), Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (1.48), was adjusted to pH 7.3 and sterilized by autoclaving. Glycine buffered saline: 20 mM glycine was used in place of phosphate salts in PBS, and adjusted to pH 4.0 before sterilization by filtration. Collagenase solution, containing 50 units/ml collagenase, 10 µg/ml soybean trypsin inhibitor and 50 mM glucose in PBS, was sterilized by filtration through 0.22 µm filters and stored at -20°C in 60 ml aliquots. DNase was prepared at 2.5 mg/ml in 1 mM HCl, and stored in 1 ml aliquots at -70°C.

Erythrocyte lysis buffer, 140 mM  $\text{NH}_4\text{Cl}$ , 17 mM Tris-HCl, pH 7.2, was sterilized by filtration.

Basal Medium (Eagle), BME, contained 1x Earle's salts, 3 g/l  $\text{NaHCO}_3$ , 20 mM D-glucose, 0.1 g/l kanamycin sulfate, 100 units/ml penicillin, 100 units/ml streptomycin sulfate, 1x BME vitamins, and augmented amino acids as given by Tarlow *et al* (1977). When used, chicken serum was at 5% (v/v) and insulin at 5  $\mu\text{g/ml}$ . Eagle's media phosphate-buffered (EPB) is identical to BME, replacing  $\text{NaHCO}_3$  with 0.2 g/l  $\text{KH}_2\text{PO}_4$ , 1.48 g/l  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , pH 7.6, and containing 4% chicken serum.

#### Hepatocyte isolation and culture

Hepatocytes were prepared by a modification of the method of Tarlow *et al* (1977). Five 12-14 day old White Leghorn chicks were sacrificed by cervical dislocation, feathers and skin removed, and the bodies rinsed with 95% ethanol. Livers were dissected and rinsed in sterile PBS at room temperature. The livers were finely minced with scissors and transferred to a sterile 250 ml flask containing 30 ml collagenase and 1 ml DNase solutions. The mince was incubated for 20 minutes at 37°C on a rotary shaker at 250 rpm. The supernatant containing suspended cells was decanted into sterile plastic tubes (Corning) and spun for 2 minutes at 500xg. The cell pellets were resuspended in 20 ml EPB and allowed to sit at room temperature.

Meanwhile, an additional 30 ml of collagenase was added to the remaining tissue mince, and the 20 minute incubation repeated. After the second collagenase digestion, the tissue mince was filtered through 100-mesh silk screen, and this filtrate was spun down and resuspended in 20 ml EPB. The cell suspensions were pooled and washed with

an additional 20 ml EPB, then resuspended in 20 ml erythrocyte lysis buffer. After 5 minutes, 20 ml EPB were added and the cells pelleted. Cells resuspended in 20 ml EPB were filtered through silk screen, and this filtrate used immediately for plating.

Hepatocyte cell suspensions were adjusted to  $2 \times 10^6$  cells/ml in warm BME supplemented with chicken serum and insulin. Cells were plated at  $10^7$  cells in 5 ml media into 60 mm plastic dishes (Falcon), and were maintained at  $37^\circ\text{C}$  under 10%  $\text{CO}_2$  in a water-jacketed incubator. Media were changed 18 hours after initial plating, and every 24 hours thereafter. Cell number was determined with a hemacytometer, and cell viability was estimated by trypan blue exclusion. Viability was 85-90% following isolation. Cells were cultured for at least two days prior to experimental manipulation, to allow for the resynthesis of surface receptors following collagenase treatment, and were used within seven days of isolation.

#### Hepatocyte treatment and membrane preparation

The sequence of chicken glucagon differs in only one residue from that of rat, pig or cow (Huang *et al*, 1987; Hasegawa *et al*, 1990), and purified chicken glucagon has equal potency with the porcine polypeptide for binding to the rat liver glucagon receptor (Huang *et al*, 1987). Binding of rat glucagon to the chicken glucagon receptor has been reported to have somewhat lower activity than the chicken hormone (Shchenkova & Kuznetsova, 1984). Purified bovine glucagon, which is identical in sequence with the porcine hormone, was used throughout this study.

Hepatocytes were generally treated with  $1 \mu\text{M}$  glucagon in culture media lacking serum for various times to induce desensitization, or with other hormones or drugs as

indicated in the figure legends. Following experimental treatment, cell monolayers were rinsed thrice with cold PBS with 0.1% BSA, and scraped from culture dishes into homogenization buffer (25 mM Na phosphate, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10  $\mu$ g/ml leupeptin, pH 7.5). Cells were broken with 10 strokes of a tight Dounce homogenizer (Kontes) on ice, and the homogenate centrifuged at 10,000xg for 30 minutes. The crude pellet was resuspended in 25 mM Na phosphate, 1 M NaCl, 5 mM EDTA, pH 7.5, and centrifuged again. This pellet was washed by resuspension in 25 mM Na phosphate, 5 mM EDTA, pH 7.5, and centrifuged. The final crude membrane pellet was resuspended using a 22G needle to a final protein concentration of 5 mg/ml in 25 mM Na phosphate, 1 mM EDTA, pH 7.5, quick frozen, and stored at -70°C until used.

#### S49 lymphoma cell culture

S49 murine lymphoma cell lines were obtained from the UCSF Cell Culture Facility. *cyc<sup>-</sup>* S49 cells lack hormone and guanine nucleotide stimulation of adenylyl cyclase due to lack of the  $\alpha_s$  mRNA and protein (Bourne *et al*, 1975; Harris *et al*, 1985). *UNC* S49 cells lack functional coupling of receptors to  $G_s$  without loss of  $G_s$  activation of adenylyl cyclase, due to a point mutation of the  $G_s$ - $\alpha$  protein (Haga *et al*, 1977; Masters *et al*, 1988). *kin<sup>-</sup>* S49 cells lack protein kinase A activity (Insel *et al*, 1975; Steinberg *et al*, 1978). Culture of S49 cells was as described by Bourne *et al* (1975). Cells were maintained at 37°C in DMEM media with 5% horse serum. S49 cell membranes were prepared by differential centrifugation following nitrogen cavitation according to the procedure of Ross *et al* (1977).

### Preparation of liver plasma membranes

Porcine livers were delivered fresh from a local abattoir, and plasma membranes prepared by a procedure based on the method of Neville (1968). 1 kg of liver was minced with scissors, passed through an Oster tissue grinder, and homogenized with 10 strokes in a loose-fitting Dounce homogenizer (Blaessig) in 1 mM potassium bicarbonate, pH 7.5. The homogenate was brought up to 10 liters in this buffer, stirred for 5 min, and filtered twice through a double layer of cheesecloth. The filtrate was centrifuged at 2000xg for 30 minutes in a Beckman J6 centrifuge. The tight pellets were resuspended in 65% sucrose to yield a final suspension in 45% sucrose. 1200 mL of the 45% sucrose suspension was poured into a Ti 15 zonal rotor and overlaid with 300 mL of 42% sucrose. The rotor was spun for 2.5 hours at 100,000xg. Membranes floating atop the sucrose cushion in the four quadrants of the rotor were removed with a plastic scoop. These membranes were diluted to 1 liter in 25 mM Na HEPES, 200 mM NaCl, pH 7.5, and washed by centrifugation at 15,000xg for 30 minutes. The membranes were washed a final time with 1 liter of 25 mM Na HEPES, 1 mM EDTA, pH 7.5, resuspended at 10-20 mg/mL in the same buffer, quick frozen, and stored at -70°C until used.

### Determination of protein

Membrane protein concentrations were determined by the procedure of Lowry *et al* (1951) using BSA as a standard. Purified preparations of G<sub>s</sub> were separated by SDS-PAGE, stained with Coomassie blue and compared to known amounts of BSA by densitometry. For the estimation of purified adenylyl cyclase, proteins were precipitated with acetone and separated by SDS-PAGE. Gels were stained with silver as described

by Wray *et al* (1981), and proteins in single bands estimated by densitometry using BSA as a standard.

#### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Proteins were separated by electrophoresis at 150 V through 8 or 15 cm polyacrylamide gels containing SDS according to Laemmli (1970). For the analysis of glucagon receptor, 10% slab gels were used and for adenylyl cyclase, 6% slab gels were run. For analysis of G proteins, 10% slab gels with acryl:bis (30:0.4) were used for higher resolution (Toutant *et al*, 1987). Stacking gels were 5% acrylamide. High molecular weight standard proteins were from Sigma and prestained standard proteins from Bethesda Research Labs. Gels were fixed with methanol:water:acetic acid (5:5:1) and stained with 0.1% Coomassie blue, or with silver according to Wray *et al* (1981), or were used immediately for electrophoretic transfer to nitrocellulose. For autoradiography, gels were dried under vacuum and exposed to Kodak X-AR X-ray film in cassettes with intensifying screens (Cronex Lightning Plus) for 4-48 hours. Densitometry of exposed X-ray films was performed using a Hoefer scanning densitometer (GS-300) connected to an IBM PC/AT running Hoefer's GS-350 data analysis software.

#### Immunoblotting

Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose sheets was carried out according to the procedure of Towbin *et al* (1979). Gels were transferred in 25 mM Tris·OH, 192 mM glycine, 20% methanol at 40 V for 16 hours or at 60 V for 2 hours in a Hoefer Trans-blot chamber in a cold room. Nitrocellulose sheets were

blocked by soaking in 50 mM Tris·HCl, 150 mM NaCl, 3% ovalbumin, 0.1% NaN<sub>3</sub>, pH 8.5 (blocking buffer) for 4-6 hours at room temperature. Nitrocellulose sheets were then exposed to antisera diluted in blocking buffer for 12-16 hours at room temperature, at the dilution indicated in particular figure legends. Excess antibodies were removed by washing the nitrocellulose sheets with moderate agitation in blocking buffer lacking ovalbumin (wash buffer) twice for 10 minutes at room temperature, then twice in wash buffer with 0.1% Lubrol, and twice again without detergent. The nitrocellulose was then reblocked by incubation with blocking buffer for 2-4 hours at room temperature. The nitrocellulose was exposed to  $1 \times 10^6$  cpm/mL [<sup>125</sup>I]-labeled goat anti-rabbit IgG or [<sup>125</sup>I]iodo-Protein A (when detecting monoclonal antibodies) for 2 hours at room temperature, and excess radioactivity removed by vigorous washing as before. Monoclonal antibody BBC-4 was directly iodinated and used without a second antibody probe. Nitrocellulose sheets were air-dried and exposed to X-ray film in cassettes equipped with Cronex Lightning Plus intensifying screens for 6-48 hours, as indicated.

#### [<sup>125</sup>I]MIG binding and crosslinking to membranes

For determination of glucagon receptor levels, hepatocyte membranes (100-500 µg/ml) were incubated for 15 minutes at 32°C in the presence of 20 mM Na phosphate (pH 7.5), 8 mM MgCl<sub>2</sub>, 1 mM EDTA, 1% (w/v) BSA, 1 nM [<sup>125</sup>I]MIG (2200 Ci/mmol), and appropriate additives, in a final volume of 100 µl. When used, insulin and glucagon were present at 10 µg/ml, and GppNHp at 100 µM. The binding reaction was terminated by removing assay tubes to ice and dilution with 3 ml of ice-cold 20 mM Na phosphate, 5 mM MgCl<sub>2</sub>, 1% BSA. The diluted samples were filtered through 0.45 µm cellulose

acetate filters (Oxoid) which had been pre-soaked in 10% BSA to reduce nonspecific binding, and immediately washed with 20 ml of ice-cold 20 mM Na phosphate, 5 mM  $\text{MgCl}_2$ , 0.1% BSA. Filter-bound radioactivity was quantitated in a  $\gamma$  counter, and specifically bound counts calculated by subtracting nonspecific from total bound counts of triplicate assay tubes.

Binding conditions for membrane crosslinking were identical, except that the reaction volume was increased to 5-20 ml. At the end of incubation, three 50  $\mu\text{l}$  aliquots of the binding reaction were removed and assayed by filtration as above. To the remainder, up to 5 ml aliquots were diluted into 20 ml of ice-cold 20 mM Na phosphate, 0.1% BSA in ultracentrifuge tubes, and spun for 20 minutes at 100,000xg. The supernatants containing unbound [ $^{125}\text{I}$ ]MIG were discarded and the membranes were resuspended in 20 ml of 20 mM Na phosphate and sedimented again. These washed pellets were resuspended in the volume of the initial binding assay, and pooled in a beaker on ice. In the dark, 20 mM HSAB in DMSO was added to the pooled membranes to a final concentration of 200  $\mu\text{M}$ , and allowed to react for 3 minutes. Membranes on ice were then irradiated for 8 minutes at 12 cm with a mercury arc lamp (Gates). Following irradiation, 1/10 volume of 50 mM Tris-HCl (pH 7.5) was added to scavenge unreacted succinimide, and the membranes were pelleted as before. Crosslinked membranes were resuspended in SDS-PAGE sample buffer for electrophoresis.

#### Adenylyl cyclase assays

Adenylyl cyclase activity was determined using 5-20  $\mu\text{g}$  of membrane protein incubated for 10 minutes at 32°C in a final volume of 50  $\mu\text{l}$ . Assays contained 25 mM

Na HEPES (pH 8.0), 1 mM EDTA, 100  $\mu$ M [ $\alpha$ - $^{32}$ P]ATP (500-2000 cpm/pmol), 1 mM [2,8- $^3$ H]cAMP (10,000 cpm/assay), and a nucleoside triphosphate regenerating system composed of 20 mM creatine phosphate, 200  $\mu$ g/ml creatine phosphokinase (100 units/mg), and 20  $\mu$ g/ml myokinase (1000 units/ml). Stimulation by hormones and NaF was measured in the presence of 6 mM  $\text{MgCl}_2$  and 100  $\mu$ M GTP. When present, glucagon,  $\text{PGE}_1$  and VIP were 20  $\mu$ g/ml, NaF was 10 mM, and (-)-isoproterenol was 200  $\mu$ M. Reactions were stopped by the addition of 100  $\mu$ l of 10 mM cAMP, 40 mM ATP, 1% SDS. cAMP was purified on Dowex AG-50 and neutral alumina columns by the method of Salomon *et al* (1974), and measured by scintillation counting. Counts were converted to enzyme activities based on recovery of [ $^3$ H]cAMP standard and correction for the measured specific activity of [ $\alpha$ - $^{32}$ P]ATP in each assay.

#### Reconstitution of *cyc*<sup>-</sup> S49 cell membrane adenylyl cyclase

$G_s$  activity of hepatocyte membrane detergent extracts was determined by reconstitution of NaF stimulation of adenylyl cyclase in *cyc*<sup>-</sup> S49 lymphoma cell membranes. Hepatocyte membranes, 5 mg protein/ml in 25 mM Tris, 1 mM EDTA, pH 7.5, were brought to 1% (w/v) Na-cholate (pH 7.5) and vortexed intermittently for 1 hour on ice. Cholate suspensions were centrifuged at 100,000g for 1 hour, and extracts containing solubilized  $G_s$  were reconstituted with *cyc*<sup>-</sup> S49 cell membranes by preincubation for 10 minutes on ice in the absence of any other assay components. Assays contained 5-10  $\mu$ g of *cyc*<sup>-</sup> S49 cell membrane protein supplemented with 0-5  $\mu$ g of cholate-solubilized hepatocyte membrane proteins, in the same conditions as described previously for adenylyl cyclase assays. NaF-stimulated adenylyl cyclase activity was

determined in 30 minute incubations in the presence of 20 mM  $\text{MgCl}_2$ . All assays in an experiment were adjusted to the final cholate concentration of the least diluted sample, in all cases less than 0.02% cholate, to control for detergent interference with the *cyc*<sup>-</sup> S49 cell membrane adenylyl cyclase. Cholate suspensions were assayed in parallel with their extracts to control for variable extraction of  $G_s$  activity.

#### Supplementation of hepatocyte or S49 cell membranes with $G_s$

Hepatocyte or S49 cell membranes were diluted to 1.3 mg/mL in 25 mM Tris, 1 mM EDTA, pH 7.5, and were incubated on ice for 10 minutes with various concentrations of purified  $G_s$  diluted in plastic tubes with 25 mM Tris, 1 mM EDTA, 20 mM 2-mercaptoethanol, 0.005% BSA, pH 7.5.  $G_s$ -supplemented membranes (20  $\mu\text{l}$ ) were assayed for adenylyl cyclase activity in a final volume of 50  $\mu\text{l}$  for 30 min as described for general adenylyl cyclase assays. Final  $G_s$  concentrations in assays were as indicated in the figure legends, as are tissue sources for purified  $G_s$  used for particular experiments.

#### In vitro desensitization of adenylyl cyclase

Hormonal desensitization of isolated membranes was carried out essentially as previously described by Iyengar *et al* (1981b). Hepatocyte membranes were incubated at 1 mg/ml in the presence of 25 mM bis tris propane (pH 7.5), 1 mM EDTA, 1 mM ATP, 100  $\mu\text{M}$  GTP, 5 mM  $\text{MgCl}_2$  and various concentrations of glucagon for 30 minutes at 32°C. Pretreated membranes were diluted to 200  $\mu\text{g}/\text{ml}$  into the usual adenylyl cyclase assay conditions, and incubated a further 10 minutes at 32°C. Accumulated cAMP was determined as described by Salomon *et al* (1974).

*In vitro* desensitization of membranes by addition of purified protein kinase A catalytic subunit was assayed in a two-stage protocol. Hepatocyte or S49 cell membranes and purified PKA were diluted in 25 mM Tris·HCl, 1 mM EDTA, pH 7.5, and mixed with adenylyl cyclase assay components as described above, omitting only stimulators and radiolabels. This mixture, containing PKA at 50 nM, was incubated with 10 min at 32°C. Triplicate aliquots of this phosphorylation mixture (40 µl) containing 10 µg of membrane protein were then added to a mixture of [<sup>3</sup>H]cAMP, [ $\alpha$ -<sup>32</sup>P]ATP and appropriate stimulators of adenylyl cyclase to a final volume of 50 µl, and incubated for another 10 min at 32°C. Accumulated cAMP was purified and quantitated by the method of Salomon *et al* (1974).

#### Pertussis toxin catalyzed ADP-ribosylation of G<sub>i</sub> proteins

Pertussis toxin (10 µg/ml) was activated by incubation with 20 mM dithiothreitol at 32°C for 20 minutes in a plastic tube. ADP-ribosylation was carried out in buffer consisting of 25 mM HEPES (pH 7.5), 10 mM thymidine, 1 mM EDTA, 1 mM ATP, 100 µM GTP, 0.1% (w/v) Lubrol-PX, and 10<sup>7</sup> cpm [<sup>32</sup>P]NAD<sup>+</sup> (5000 cpm/pmol) in a 50 µl final volume. 0.25-10 µg membrane protein was utilized per assay. ADP-ribosylation was initiated by addition of 10 µl of membrane proteins and 10 µl (100 ng) of activated pertussis toxin to each assay tube. Following incubation for 30 minutes at 32°C, reactions were removed to ice and diluted with 1 ml cold 20% (w/v) trichloroacetic acid. After 10 minutes on ice, samples were pelleted at 10,000xg for 15 min, and the radioactive supernatant discarded. The precipitate was washed with 1 ml diethyl ether, dried under a stream of N<sub>2</sub>, and resuspended in SDS-PAGE sample buffer for electrophoresis.

### Purification of G proteins

$G_s$ ,  $G_i$ , and  $\beta\gamma$ -subunits were purified from human erythrocytes as described by Codina *et al* (1984b), and from rabbit liver as described by Bokoch *et al* (1984) except that all buffers following the extraction step contained 30% ethylene glycol.  $G_s$  was followed as *cyc*<sup>-</sup> S49 cell membrane adenylyl cyclase reconstituting activity, and the  $G_i$  proteins as pertussis toxin substrates following the heptylamine chromatography. Following the second DEAE-Sephacel step, proteins were concentrated on 2cc hydroxylapatite columns. The hydroxylapatite eluates were diluted and loaded onto 0.5cc DEAE-Sephacel columns, washed free of phosphate buffer, and eluted in a buffer containing 10 mM Na HEPES, 1 mM EDTA, 20 mM 2-mercaptoethanol, 0.1% Lubrol, 30% ethylene glycol, 200 mM NaCl, pH 8.0. The  $\beta\gamma$ -subunits were obtained resolved from  $\alpha$ -subunits on the second DEAE-Sephacel chromatography, diluted and loaded on a 0.5cc DEAE column. The  $\beta\gamma$ -subunits were eluted in the above buffer. All G proteins were stored at -70°C until used. G protein concentrations were determined by densitometry of bands in Coomassie blue-stained SDS-PAGE slab gels, using BSA as a standard.

### Phosphorylation of purified proteins with protein kinase A

Phosphorylation reactions contained 20 mM K phosphate, pH 7.5, 6 mM  $MgCl_2$ , 1 mM EDTA, 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (20,000 cpm/pmol) in a final volume of 50  $\mu$ l, and were initiated by addition of PKA to 5 nM. Sample proteins were added to make at least 100 nM concentration. Assays were incubated for 30 min at 32°C, and stopped by addition of 1 ml 20% TCA and 5  $\mu$ g BSA as carrier. Samples were pelleted at 10,000xg

and washed again with 20% TCA. The pellets were then washed with diethyl ether, dried under N<sub>2</sub>, and resuspended in SDS-PAGE sample buffer for electrophoresis.

#### [ $\alpha$ -<sup>32</sup>P]ATP crosslinking to purified adenylyl cyclase

Purified preparations of adenylyl cyclase were incubated with 1  $\mu$ M [ $\alpha$ -<sup>32</sup>P]ATP (50 Ci/mmol) in the presence or absence of 20-fold excess unlabeled ATP or GTP. The assay contained 25 mM HEPES (pH 7.5), 1 mM EDTA, 10 mM MnCl<sub>2</sub> and 50  $\mu$ M forskolin (activating conditions), or 25 mM Tris (pH 8.5), 5 mM EDTA (deactivating conditions). Assays were incubated for 2 minutes at room temperature, then irradiated on ice from 15 cm with a UV lamp (Gates) for 10 minutes. 1  $\mu$ g BSA was added, and proteins precipitated with 10% TCA. The protein pellet was washed with diethyl ether, dried under N<sub>2</sub>, and resuspended in sample buffer for SDS-PAGE.

For enzymatic analysis of the glycosylation state of adenylyl cyclase, the ether-washed ATP-crosslinked protein pellet was instead resuspended in 30  $\mu$ L of 25 mM HEPES (pH 6.0), 1 mM EDTA, 5 mM MgCl<sub>2</sub>. Glycopeptidase F (0.2 U) was added, and the reaction continued overnight at 30°C in a final volume of 40  $\mu$ L. The reaction was stopped by addition of concentrated sample buffer, and the proteins separated by SDS-PAGE for autoradiography.

#### DNA amplification by the polymerase chain reaction

DNA sequences (cDNAs from a  $\lambda$  phage library, or first strand cDNA prepared from poly(A) RNA) were amplified by the polymerase chain reaction using heat-stable DNA polymerase from *Thermus aquaticus* in an automated thermal cycler (Saiki *et al*,

1985; Innes *et al*, 1990). Reactions (100  $\mu$ l) contained Taq polymerase buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% gelatin, 0.1% Triton X-100), 500 nM of each primer, 200  $\mu$ M of each dNTP (neutralized), 2.5 units of Taq polymerase (Promega).  $MgCl_2$  concentrations were varied from 1-3 mM as indicated. When amplifying cDNA in  $\lambda$  phage,  $10^8$  pfu were used per reaction. Reactions were covered with 100  $\mu$ l of light mineral oil to prevent evaporation, and subjected to 30 cycles of denaturation (95°C 1 min), annealing (35-60°C 1 min as indicated), and extension (72°C 3 min) followed by a final 10 min extension in a Perkin-Elmer Cetus thermal cycler. Reactions were extracted with 150  $\mu$ l chloroform, the aqueous phase dried in a Speed-vac, resuspended in sample buffer, and subjected to electrophoresis in NuSieve agarose gels containing TAE buffer and 0.5  $\mu$ g/ml ethidium bromide. DNA bands were visualized by UV transillumination, and appropriate size DNA products excised with a razor. DNA was recovered from agarose using Gene-Clean (Bio 101), or by directly spinning DNA and buffer from gel slices through a plug of siliconized glass wool in a punctured microfuge tube into a larger tube, followed by phenol extraction. Purified DNA was directly sequenced from end-labeled primers using Taq polymerase (TAQuence, US Biochemicals). Purified DNA was digested with rare-cutting restriction enzymes whose sites were added to the 5' ends of the DNA primer sequences, and the products directionally ligated into pBS (digested with the same enzymes) using phage T4 DNA ligase (6 U) in 20  $\mu$ l reactions overnight at 4°C. Ligated DNA was transfected into competent XL1 Blue *E. coli* (Stratagene) by 42°C heat-shock for 45 seconds. Transfected bacteria were spread on LB plates with tetracycline, ampicillin, IPTG, and Blueo-gal. Plasmid DNA minipreps were prepared for selected colonies as described for restriction

mapping, DNA sequencing, and isolation of insert DNA.

### Screening of cDNA libraries

Bovine brain cDNA libraries (1-2 kb, 2-4.4 kb, >4.4 kb size-selected fractions) in  $\lambda$ ZAP II (Vogel *et al*, 1988) were kindly provided by Dr. Richard Dixon (Merck, West Point, PA). Rat liver cDNA in  $\lambda$ ZAP II was purchased from Stratagene, and in  $\lambda$ gt11 from Clontech.  $\lambda$  phage were grown on XL1 Blue strain *Escherichia coli* on LB agar plates supplemented with 12  $\mu$ g/mL tetracycline ( $\lambda$ ZAP II) or on Y1090 strain in the presence of 50  $\mu$ g/ml ampicillin ( $\lambda$ gt11). Initial platings of the libraries were at 50,000 phage/150 mm plate using top agarose (0.7%), and subsequent platings used 100 mm plates with top agar. Phage plate replicas were prepared on duplicate nitrocellulose circles for 2 minutes; orientation was marked by stabbing with an India ink-filled syringe. Nitrocellulose filters were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, and rinsed twice in 2xSSC buffer, all for 5 min each. Filters were baked for 2 hours at 80°C under vacuum to link DNA to nitrocellulose. Filters were prehybridized for 2-4 hours at room temperature in 6xNET (0.9 M NaCl, 6 mM EDTA, 90 mM Tris-HCl, pH 8.3), 5x Denhardt's solution, 0.1% SDS, and 100  $\mu$ g/mL sonicated, denatured salmon sperm DNA. Hybridization was performed by adding radiolabeled probe (denatured by boiling) directly to the prehybridization solution and incubating the mixture for 16 hours at an appropriate temperature in heat-sealed bags. Hybridized filters were washed with 6xSSC three times at room temperature, and three times for 5 min at the hybridization temperature before exposure to X-ray film in cassettes with intensifying screens. Signals were considered significant only if present

on duplicate filters, and positive phage were selected with toothpicks into SM media (25 mM Tris·HCl, 8 mM MgSO<sub>4</sub>, 100 mM NaCl, 0.1% gelatin, pH 7.5) containing 2% chloroform to kill bacteria.  $\lambda$  phage positive for a given probe were purified by 3-4 rounds of limiting dilution and rehybridization selection until all phage on a dilution plate gave positive hybridization signals.

#### Subcloning and characterization of cDNA clones

Purified  $\lambda$ ZAP II phage clone inserts were subcloned into pBlueScript (pBS) by phagemid excision upon co-infection of XL1 Blue *E. coli* with R408 helper phage according to Short *et al* (1988), except that only 10<sup>8</sup> pfu helper phage were used. Phagemids were collected from the co-infection culture supernatant following five hour incubation, and heated to 70°C for 20 min to inactivate parent  $\lambda$  phage. Phagemids were used to infect fresh XL1 Blue cells, and dilutions plated on LB plates supplemented with 12  $\mu$ g/mL tetracycline, 50  $\mu$ g/ml ampicillin, 1 mM IPTG, and 200  $\mu$ g/ml Blueo-gal were grown at 42°C to reduce helper phage contamination.

Colonies containing pBS with inserts were transferred to LB master plates and grown in liquid culture overnight at 37°C in 5 ml LB with tetracycline and ampicillin. DNA minipreps were prepared by the alkaline lysis method (Sambrook *et al*, 1989). Cells were pelleted and resuspended in 200  $\mu$ l of 25 mM Tris·HCl (pH 8.3), 10 mM EDTA, 50 mM glucose, 10  $\mu$ g lysozyme, and incubated for 5 min at room temperature. Bacteria were lysed by addition of 400  $\mu$ l of 0.2 N NaOH, 1% SDS with gentle mixing for 5 min on ice. 300  $\mu$ l of 3 M K acetate (pH 4.8) was added for 5 min on ice, and cell debris removed by centrifugation at 5000xg. Supernatant (2x 400  $\mu$ l) was removed to fresh

tubes and extracted first with phenol:SEVAG (1:1) and then with SEVAG (chloroform:isoamyl alcohol, 24:1). To the extracted aqueous phases was added 800  $\mu$ l ethanol, DNA was precipitated for 15 min at room temperature, and collected by centrifugation at 10,000xg for 10 min. Pellets were resuspended in 270  $\mu$ l water, pooled, and precipitated on ice for 1 hour after addition of 260  $\mu$ l of 20% PEG (8000), 2 M NaCl. Pellets collected by centrifugation were rinsed with 70% ethanol, dried under vacuum, and resuspended in TE buffer for restriction analysis or DNA sequencing.

#### Nested PCR screening of cDNA libraries

Individual clones extending beyond the end of an already determined sequence were amplified from complete  $\lambda$  phage cDNA libraries using a two-stage variation of the "anchored" polymerase chain reaction (Frohman *et al*, 1988; Innes *et al*, 1990; Gibbons *et al*, 1991). Instead of a standard anchored PCR, where a known sequence is added to the end of a template to allow amplification across an unknown sequence, I have taken advantage of the known sequences of the  $\lambda$  phage vector at the ends of clones in cDNA libraries to amplify individual clone sequences from the library. This procedure has allowed the rapid screening of several cDNA libraries at once, as well as selection of the longest clones from a mixture of several positive DNAs. Commercial rat liver cDNA libraries previously screened by hybridization (in  $\lambda$ ZAP II from Stratagene; in  $\lambda$ gt11 from Clontech) were also screened by this nested PCR technique. A rat kidney cDNA library in  $\lambda$ gt10 was obtained from Dr. Kevin Lynch (Burnham *et al*, 1987) and another in  $\lambda$ ZAP II from Stratagene were also used. Rat hepatocyte (in  $\lambda$ ZAP II, gift of Dr. Miles Houslay) and rat heart (in  $\lambda$ gt11, gift of Dr. Mark Shields) cDNA libraries were also

screened, but did not yield the longest clones in any reaction, so these products have not been further analysed.

Pairs of oligonucleotide primers which match sequences in the  $\lambda$  phage DNA immediately flanking the multiple cloning site were prepared for  $\lambda$ ZAP II,  $\lambda$ gt10 and  $\lambda$ gt11 vectors (ie., T3 and T7 promoter sequencing primers for  $\lambda$ ZAP II). From the known sequence near the end of a clone, two independent, nested oligonucleotide primers were also synthesized. The clone primers have their 3' ends facing the unknown end of the sequence. An initial PCR amplification reaction is performed using one of the appropriate vector primers and the outer of the two clone primers. This will amplify all clones in the library which are in the proper orientation relative to the phage primer, as well as many other nonspecific products (since all phage have sequences matching the vector primer). A complementary reaction is also performed using the same clone primer but the second vector primer in order to amplify insert sequences in the opposite orientation relative to the phage cloning site. Products from both of these initial reactions (1  $\mu$ l) are reamplified using the same vector primers but the nested clone primer. Since nonspecific DNAs would be unlikely to contain the second sequence, most products are specific (and therefore of interest if they are long enough to contain new sequences). A portion of these secondary reactions are separated on agarose gels and hybridized to a probe based on the known sequence which is nested within the second PCR primer. Positively hybridizing products are cut from agarose gels and subcloned into pBlueScript by taking advantage of the EcoR1 site amplified from the vector multiple cloning site on one side and an EcoR1 site added into the second PCR primer sequence.

### DNA sequencing

Double-stranded plasmid DNA (2 µg/primer) was denatured by treatment with 0.2 N NaOH for 5 min. Ammonium acetate (pH 5.2) was added to 300 mM, and DNA precipitated with 2 volumes of ethanol. Denatured plasmid DNA was sequenced using modified T7 DNA polymerase (Sequenase, US Biochemical) and [ $\alpha$ -<sup>32</sup>P]dATP (650 Ci/mmol). DNA was resuspended in Sequenase reaction buffer with 1 pmol primer and annealed by heating to 65°C for 5 min and cooling to ambient temperature over 1 hour. pBS was sequenced from T7 or T3 primers, or from insert-specific primers. Annealed template-primer was extended for 5 min at room temperature and terminated for 5 min at 37°C according to the manufacturer's protocol. Reactions were denatured by heating to 70°C prior to loading on 6 or 8% polyacrylamide sequencing gels (0.4 mm to 0.8 mm wedge) containing 7 M urea and TBE buffer. Gels were run at 1500-2000 V, and dried under vacuum for autoradiography. Sequences were read manually, and consensus sequences determined and analyzed using the PC Gene DNA analysis software (Intelligenetics). Ambiguous sequences and compressions were resolved by resequencing in the presence of 7-deaza-dGTP or dITP instead of dGTP.

### RNA preparation

Total RNA was extracted and purified from tissues and cultured cells using the guanidinium/CsCl<sub>2</sub> method (Chirgwin *et al*, 1979). 2 grams of tissue was homogenized in 15 ml of buffer (5 M Guanidinium thiocyanate, 50 mM Tris·HCl (pH 7.5), 10 mM EDTA, 5 % 2-mercaptoethanol) using a Polytron at setting "4" for 1 minute. Sarkosyl (20%) was added to 0.5% and the homogenate spun at 4000 rpm for 15 min in a

Beckman J-6B centrifuge. The supernatant was drawn through a 22G needle to shear cellular DNA and spun a second time in a fresh tube. The resulting homogenate was carefully layered atop a cushion of 5.7 M CsCl, 10 mM EDTA in an ultracentrifuge tube (20 ml in an SW28 tube), and spun at 100,000g for 18 h. The gradient was carefully aspirated to within 1 cm of the bottom of the tube, and the remaining liquid decanted in a quick motion. The RNA pellet was dried for 5 min by inverting the tube, and then washed with 1 ml of cold 70% ethanol. The pellet, after a second drying period, was resuspended in 3 ml of 10 mM Tris·HCl, 5 mM EDTA, 1% SDS, and extracted with phenol/SEVAG (1:1). The aqueous phase was removed to a fresh tube, and RNA precipitated at -20°C following addition of 300 µl of 3 M Na acetate (pH 5.2) and 9 ml of ethanol. Liver RNA was subjected to a second precipitation in 3 M NaCl at 4°C overnight to remove glycogen. RNAs were resuspended in 500 µl DEPC-treated H<sub>2</sub>O and the concentration determined spectrophotometrically. Total RNAs were stored as 75% ethanol suspensions at -20°C.

Poly(A) RNA was selected from total RNA using oligo(dT)-cellulose chromatography (Sambrook *et al*, 1989), using spun columns (Pharmacia) according to the manufacturer's protocol. Partially purified poly(A) RNA (after a single passage), was concentrated by ethanol precipitation and stored at -70°C.

#### Preparation of first strand cDNA

First strand cDNA for PCR amplification was synthesized using MMLV reverse transcriptase (SuperScript, GIBCO/BRL). One µg of poly(A) RNA was added to 100 pmol of oligonucleotide primer to give 10 µl, denatured by heating to 75°C for 10 min,

and annealed by cooling on ice. The oligonucleotide primer contained (dT)<sub>18</sub> with a 5'-end 25 base sequence which could be used for PCR amplification and subcloning, or was specifically complementary to a sequence in a mRNA of interest. The cDNA extension reaction contained 10 mM DTT, 40 U RNasin, 1x SuperScript reaction buffer, 500  $\mu$ M each dNTP and 200 U MMLV reverse transcriptase in 20  $\mu$ l, and was incubated for 1 hour at 42°C. Reactions were diluted with water and stored at -20°C.

## CHAPTER 3

### GENERAL CHARACTERISTICS OF GLUCAGON-INDUCED DESENSITIZATION AND THE HOMOLOGOUS REGULATION OF THE GLUCAGON RECEPTOR

Studies of isolated liver cell function have most often utilized non-proliferating primary cultures of rat hepatocytes obtained by the collagenase perfusion method of Berry and Friend (1969). Rat hepatocytes in primary culture, however, are often difficult to maintain for extended periods and undergo de-differentiation or loss of liver-specific functions (Bissell & Guzelian, 1980, Bayad *et al*, 1991). A chick hepatocyte primary culture system developed in the laboratory of M. D. Lane has been shown to maintain hormone sensitivity and liver specific protein secretion following culture for periods of up to 1 week (Tarlow *et al*, 1977). Livers dissected from 2 to 3 week-old chicks are digested externally with collagenase, and the resulting hepatocytes maintained in non-proliferating culture. These hepatocytes retain the ability to synthesize fatty acids in response to insulin, and this synthesis is dramatically inhibited by glucagon or by cAMP analogs (Watkins *et al*, 1977). Further, these cells maintain an insulin receptor complement which can be down-regulated by prolonged exposure to insulin and which recovers following removal of the hormone (Krupp & Lane, 1981). Therefore these cells appeared to be a potentially useful model system for studying regulation of the glucagon-sensitive adenylyl cyclase system.

### Chick Hepatocyte Glucagon-Stimulated Adenylyl Cyclase System

Cultured avian hepatocytes can be maintained in non-proliferating primary culture for more than one week without apparent de-differentiation, with the major limitation being overgrowth by contaminating epithelial cells later in the culture. The hepatocytes maintain a stable membrane potential for at least one week, and contain voltage dependent  $K^+$ -channels (Marchetti *et al*, 1988). Chick hepatocytes also continue to express functional components of the glucagon-stimulated adenylyl cyclase system following isolation. Crude membranes from cultured hepatocytes exhibit GTP- and  $Mg^{2+}$ -dependent glucagon-stimulated adenylyl cyclase activity and  $Mg^{2+}$ -dependent, GTP-sensitive [ $^{125}I$ ]MIG binding activity (*see below*), indicating that these cells continue to express all the protein components required for hormonal stimulation of adenylyl cyclase. These activities are comparable to those from membrane preparations from intact chicken liver (*e.g.*, Padrell *et al*, 1987).

The glucagon receptor can be visualized by crosslinking [ $Tyr^{10-125}I$ ]mono-iodoglucagon ([ $^{125}I$ ]MIG) specifically-bound to the glucagon receptor using the heterobifunctional crosslinking agent N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) under UV illumination (Johnson *et al*, 1981; Iyengar & Herberg, 1984). [ $^{125}I$ ]MIG bound to crude membrane preparations was crosslinked by treatment with HSAB, and proteins analyzed by SDS-PAGE and autoradiography as shown in Figure 3-1, panel A. The crosslinked glucagon receptor from cultured chick hepatocytes exhibits the same apparent size (63 kDa) as the receptor in chicken liver membranes as described previously (Padrell *et al*, 1987). Crosslinking of [ $^{125}I$ ]MIG to this 60 kDa hepatocyte membrane protein is not blocked in the presence of excess insulin, is reduced by addition of GTP and is

Figure 3-1. Crosslinking of [ $^{125}$ I]MIG to the chick hepatocyte glucagon receptor protein. *Panel A*: Membranes from untreated hepatocytes were incubated with 1 nM [ $^{125}$ I]MIG alone or in the presence of 1  $\mu$ M insulin, 10  $\mu$ M GTP or 1  $\mu$ M glucagon, as indicated. Membranes were washed to remove excess label, and treated with 20 mM HSAB in the dark for 3 min, followed by UV illumination for 8 min. An autoradiograph of membrane proteins separated by SDS-PAGE is shown, with standards as indicated on the left. The 63 kDa glucagon receptor band is indicated by the arrow.

## Chick Hepatocyte Membrane-Crosslinking

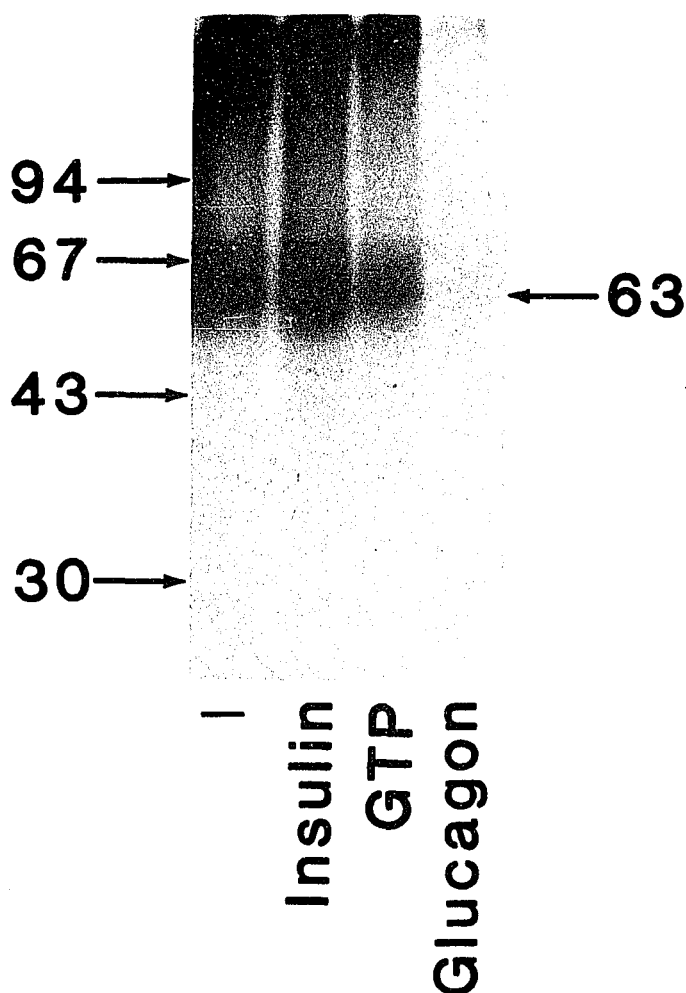
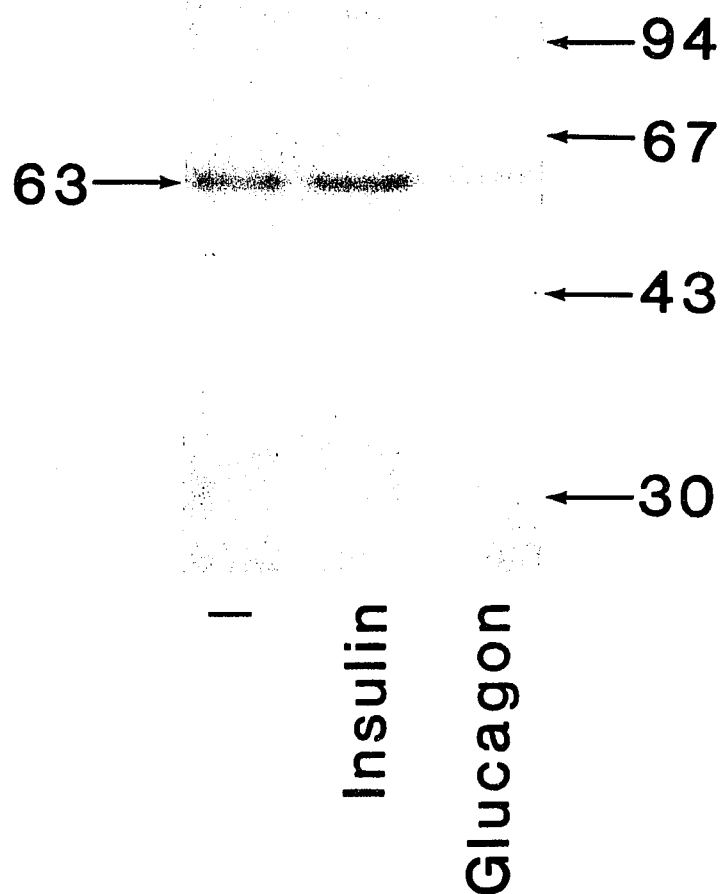


Figure 3-1, continued. Crosslinking of [ $^{125}$ I]MIG to the chick hepatocyte glucagon receptor protein. *Panel B*: Intact hepatocytes were incubated with [ $^{125}$ I]MIG alone or in the presence of insulin or glucagon, as indicated. Cells were washed and treated with 20 mM HSAB in the dark, rinsed, and treated with UV light prior to homogenization and membrane preparation. An autoradiograph of SDS-PAGE separated membrane proteins is shown, with standard marker proteins indicated on the left.

## Chick Hepatocyte Cell-Surface Receptors



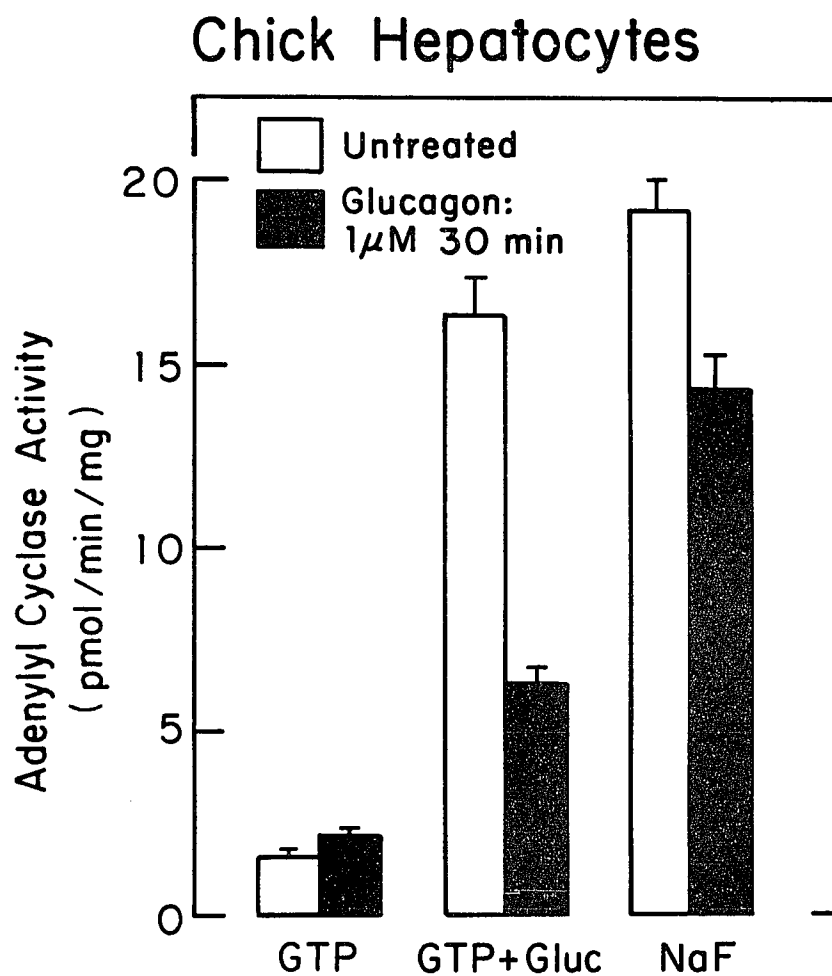
blocked by unlabeled glucagon, as expected for the glucagon receptor. The presence of the receptor protein on the external face of the cell membrane is demonstrated in Figure 3-1, panel B. [ $^{125}$ I]MIG was bound to the surface of intact cells in the presence and absence of excess insulin or glucagon, and bound hormone was crosslinked to the glucagon receptor by treatment with HSAB. Glucagon-sensitive labeling of this 63 kDa receptor protein band is observed following cellular homogenization, membrane preparation, SDS-PAGE and autoradiography.

#### Characteristics of the Desensitization Response

Treatment of chick hepatocytes for 30 minutes with 1  $\mu$ M glucagon produces a characteristic pattern of reductions in stimulated adenylyl cyclase activities of treated cell membranes as compared to untreated control cell membranes. As shown in Figure 3-2 for one experiment, glucagon stimulation is reduced to a greater extent than is NaF stimulation of adenylyl cyclase. In individual experiments, glucagon stimulation of adenylyl cyclase is generally reduced by 45-70% and NaF stimulation by 15-30% compared to untreated cell membranes.

Experiments summarized in Table 3-1 indicate that 30 minute treatment of hepatocytes with 1  $\mu$ M glucagon induces an average 60% reduction of glucagon stimulated adenylyl cyclase activity of isolated cell membranes. Stimulation of adenylyl cyclase by several other hormones and by NaF is similarly reduced following glucagon treatment, by an average of 20%. Since stimulation of adenylyl cyclase by various hormones other than glucagon (as well as receptor-independent stimulation of adenylyl cyclase by NaF) are all proportionately reduced following treatment with glucagon, chick

Figure 3-2. Adenylyl cyclase activity of untreated and glucagon-treated chick hepatocyte membranes. Parallel hepatocyte cultures were incubated in the absence or presence of 1  $\mu$ M glucagon for 30 min prior to cell homogenization and membrane preparation. Membrane adenylyl cyclase activity stimulated by GTP, GTP/glucagon and by NaF are compared for untreated (open bars) and glucagon-treated (closed bars) hepatocytes from one experiment. The reduction in adenylyl cyclase activity in the glucagon-treated membranes is due to desensitization.



hepatocytes appear to undergo some form of heterologous desensitization of the adenylyl cyclase system. The similarity in the extent of this heterologous reduction implicates a common regulatory mechanism, while the reduction in NaF-stimulation indicates that this heterologous desensitization involves some post-receptor modulations. The proportionately greater decline in glucagon stimulated adenylyl cyclase activity indicates that a second, glucagon-specific regulatory mechanism is also operating in chick hepatocytes. Thus, the chick hepatocyte adenylyl cyclase system appears to exhibit both homologous- and heterologous-type desensitizations.

To determine the concentration of glucagon required to elicit desensitization, hepatocytes were incubated in the presence of varying concentrations of glucagon for 30 minutes prior to the preparation of membranes. As shown in Figure 3-3, loss of glucagon- and NaF-stimulated adenylyl cyclase activities occur in a concentration dependent manner, with half-maximal effects at glucagon concentrations between 1 and 30 nM. Additionally, there is a parallel dose-dependent loss of specific [ $^{125}$ I]MIG binding sites as measured at a fixed agonist concentration, adding further evidence for an alteration at the level of the glucagon receptor.

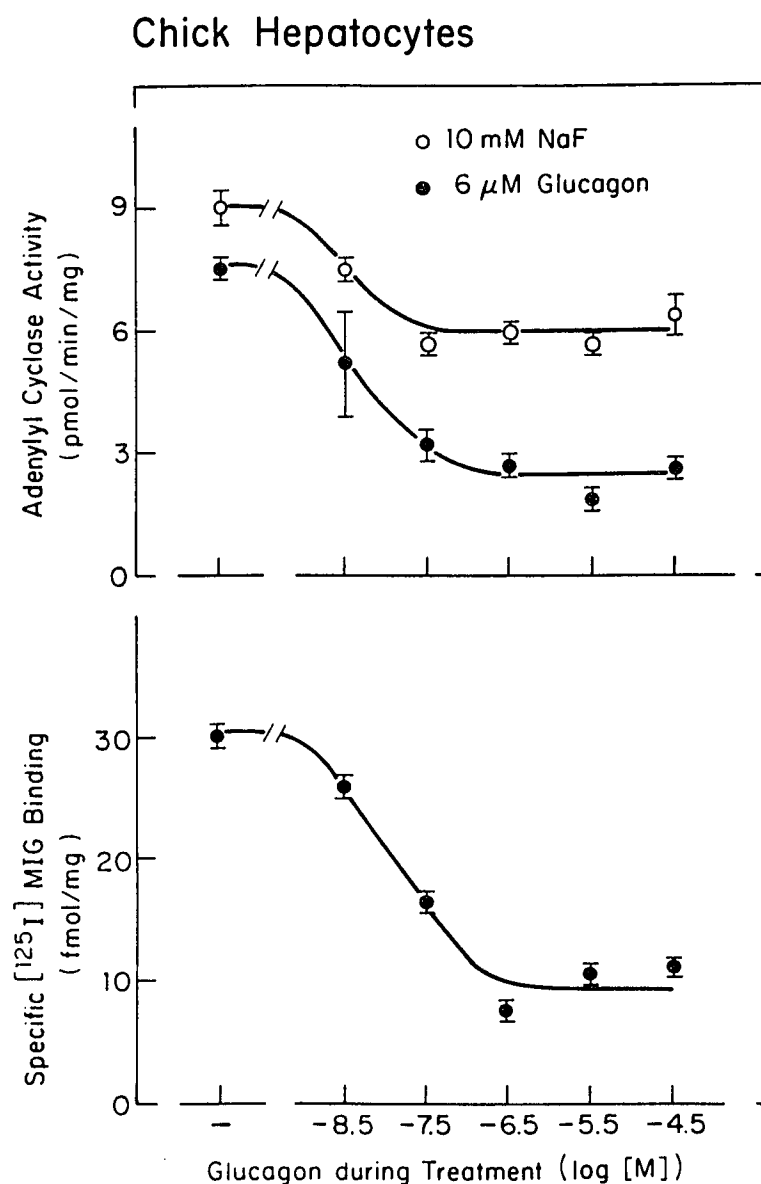
#### Kinetics of the Desensitization Process

The time-course of treatment of hepatocytes with glucagon (Figure 3-4) reveals that the desensitization response is quite rapid and exhibits at least two distinct phases. The adenylyl cyclase responsiveness to 10 mM NaF by membranes prepared from hepatocytes treated with 1 or 30  $\mu$ M glucagon for the indicated times is less than the NaF-stimulated activity in membranes from untreated cells, with a maximal 30% decrease

Table 3-1. Effect of glucagon treatment on hepatocyte membrane adenylyl cyclase activity stimulated by various effectors. Hepatocytes were incubated in the absence or presence of 1  $\mu$ M glucagon for 30 min prior to homogenization. Membrane adenylyl cyclase activity in the presence of GTP and the indicated stimulators was measured, and the percentage decrease observed following glucagon treatment for paired membranes was obtained. The average  $\pm$  SEM decrease observed from assaying the number of independent membrane preparations indicated in parentheses is indicated for each stimulator.

| Stimulator during assay       | % decrease in stimulated<br>adenylyl cyclase activity<br>following glucagon treatment |
|-------------------------------|---------------------------------------------------------------------------------------|
| glucagon                      | 59.3 $\pm$ 3.7 (21)                                                                   |
| (-)-isoproterenol             | 20.4 $\pm$ 2.7 (7)                                                                    |
| prostaglandin E <sub>1</sub>  | 25.2 $\pm$ 2.9 (7)                                                                    |
| vasoactive intestinal peptide | 22.7 $\pm$ 2.6 (3)                                                                    |
| NaF                           | 21.4 $\pm$ 2.2 (21)                                                                   |

Figure 3-3. Dose-dependence of glucagon-induced desensitization. Hepatocyte cultures were incubated in the presence of the indicated concentrations of glucagon for 30 min prior to homogenization and membrane preparation. Top Panel: Adenylyl cyclase activity of treated cell membranes stimulated by NaF (open) or by glucagon (closed circles). Glucagon-treatment reduces the adenylyl cyclase stimulation by both activators half-maximally at a concentration near 3 nM. Bottom Panel: The same membranes were assayed for specific glucagon binding sites using 1 nM [ $^{125}$ I]MIG in the absence and presence of 10  $\mu$ g/ml unlabeled glucagon. Half-maximal loss of glucagon binding activity occurs following hepatocyte treatment with 10 nM glucagon.



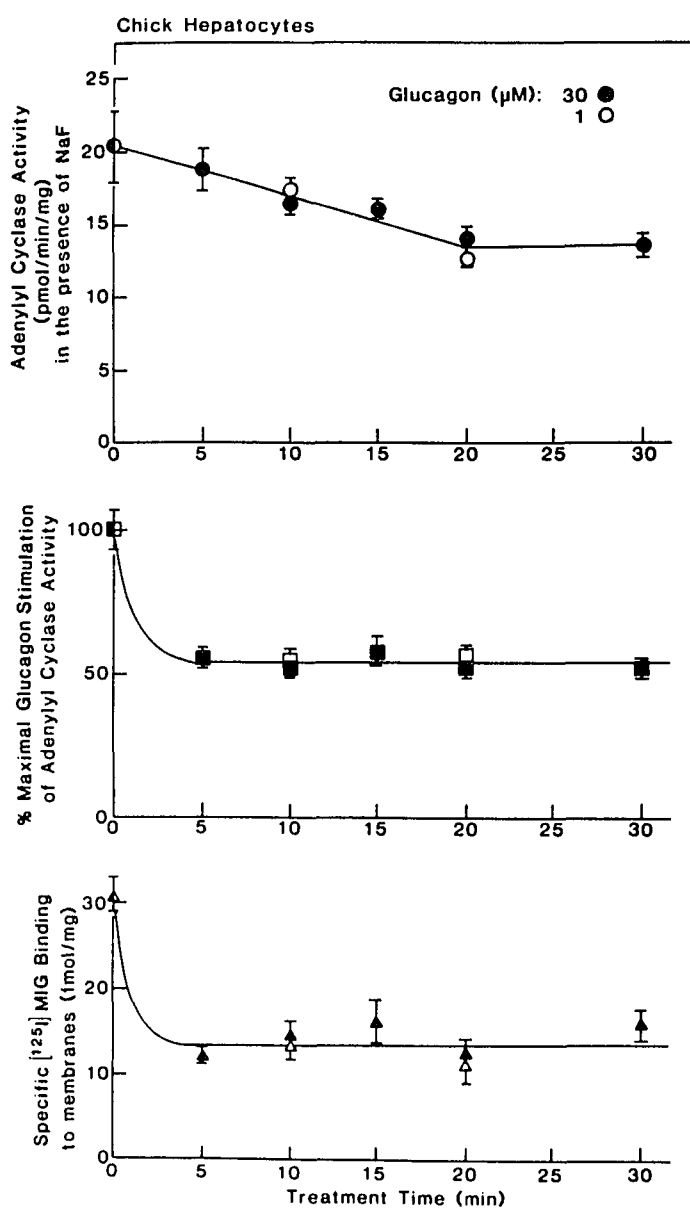
occurring after 30 minutes. Full development of this heterologous desensitization requires 20-30 minutes (top panel), and longer treatment yields no further reduction in the stimulation of adenylyl cyclase activity (not shown). The center panel demonstrates a rapid (<5 minutes) loss of glucagon-stimulated adenylyl cyclase activity, which occurs *prior* to the full onset of heterologous desensitization, as shown in the top panel. Glucagon-stimulated adenylyl cyclase activity is shown normalized by the NaF-stimulated activity at each point, since any decrease in NaF-stimulated activity (through  $G_s$ ) will also be reflected in a similarly decreased activity of other stimulatory pathways (receptors) that work through  $G_s$ . This normalized plot allows a separation of the slower, nonspecific or heterologous phase of desensitization from the initial, glucagon-specific or homologous phase.

Specific [ $^{125}$ I]MIG binding to the same membranes, measured at 1 nM [ $^{125}$ I]MIG, reveals the rapid loss of glucagon binding activity (lower panel). This loss occurs essentially in parallel with the loss of glucagon-stimulated adenylyl cyclase activity. Membranes from treated cells appear to contain 50% less binding activity than those from untreated cells, when assayed at 1 nM [ $^{125}$ I]MIG. The coordinate loss of glucagon binding activity and of glucagon stimulation of adenylyl cyclase implicates some change at the glucagon receptor as a cause of this initial homologous phase of desensitization.

#### In vitro desensitization

Crude membranes from untreated hepatocytes were incubated with various concentrations of glucagon in the presence of ATP, GTP, and  $Mg^{2+}$ , and then assayed for

Figure 3-4. Time-dependence of glucagon-induced desensitization. Hepatocytes were incubated in the presence of 1  $\mu\text{M}$  (open) or 30  $\mu\text{M}$  (closed symbols) glucagon for the indicated periods of time prior to homogenization and membrane preparation. Top Panel: Membrane adenylyl cyclase activity stimulated by NaF. Middle Panel: Membrane adenylyl cyclase activity stimulated by glucagon. Activities are normalized by the NaF-stimulated adenylyl cyclase activity measured at each time point, in order to eliminate any components of the activity loss due to common (post-receptor) alterations, since any decreased NaF stimulation (through  $G_s$ ) will also be reflected in a lower activity stimulated by any hormone which also stimulates  $G_s$ . Bottom Panel: Specific [ $^{125}\text{I}$ ]MIG binding activity of the same membranes, measured at 1 nM tracer. Note the distinct time-courses for the loss of activity measured by NaF and glucagon, and the similarity of the time-course for loss of glucagon-stimulated adenylyl cyclase and glucagon binding activity.



adenylyl cyclase activity. The experiment in Figure 3-5 shows the effect of 30 minute pretreatment with varying concentrations of glucagon. There is no measurable loss of NaF-stimulated adenylyl cyclase activity under these conditions, indicating that the loss of NaF-stimulated adenylyl cyclase activity in intact cells requires some factors not present in crude membrane fractions. Glucagon stimulation, however, is reduced by 35% when membranes are incubated with 10  $\mu$ M glucagon. This desensitization phenomenon in membranes is dependent on the presence of ATP, since the non-hydrolyzable ATP analog AppNHp does not support desensitization (data not shown). Despite numerous attempts, this response has not been observed in more highly purified membrane preparations, implying that loss of some required factor(s) ( $\beta$ ARK/ $\beta$ arrestin?) may be occurring with further purification.

#### Regulation of the Glucagon Receptor

To determine the nature of the reduction in glucagon binding activity, the [ $^{125}$ I]MIG binding activities of membranes from untreated and glucagon-treated cells were determined at several agonist probe concentrations and plotted according to Scatchard (1949). As shown in Figure 3-6 (top panel), membranes from untreated hepatocytes display an apparently homogeneous receptor state, with an affinity near 1 nM. Membranes from hepatocytes treated with glucagon for 5 minutes, however, exhibit apparently biphasic Scatchard plots, indicating the presence of both high and low affinity [ $^{125}$ I]MIG binding sites. The high affinity site appears similar to that seen in membranes from untreated hepatocytes, except that the apparent number of sites is reduced by 50% upon glucagon treatment.

Figure 3-5. *In vitro* desensitization of hepatocyte membranes by glucagon. Untreated hepatocyte membranes (1 mg/ml) were preincubated at 32°C for 30 min in the presence of the indicated concentrations of glucagon (and 25 mM bis tris propane, 1 mM EDTA, 1 mM ATP, 0.1 mM GTP, pH 7.5). Membranes were diluted five-fold into standard adenylyl cyclase assays and the incubation continued for 10 min. Adenylyl cyclase stimulation was measured in the presence of glucagon (open circles) and NaF (closed circles).

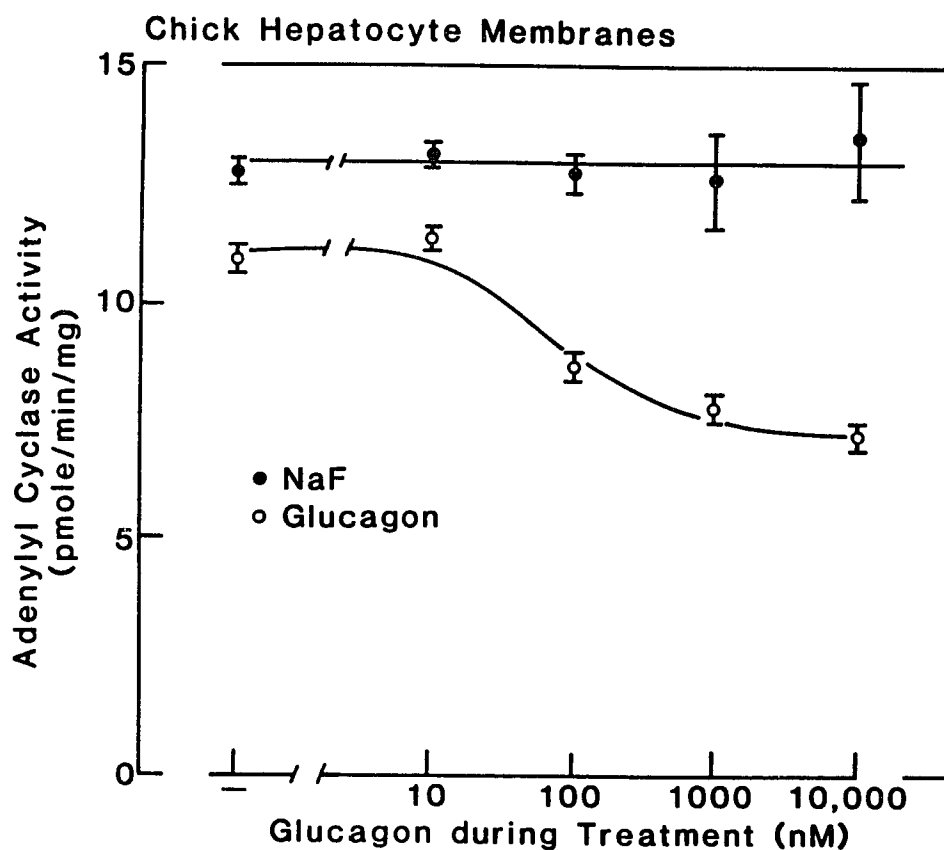
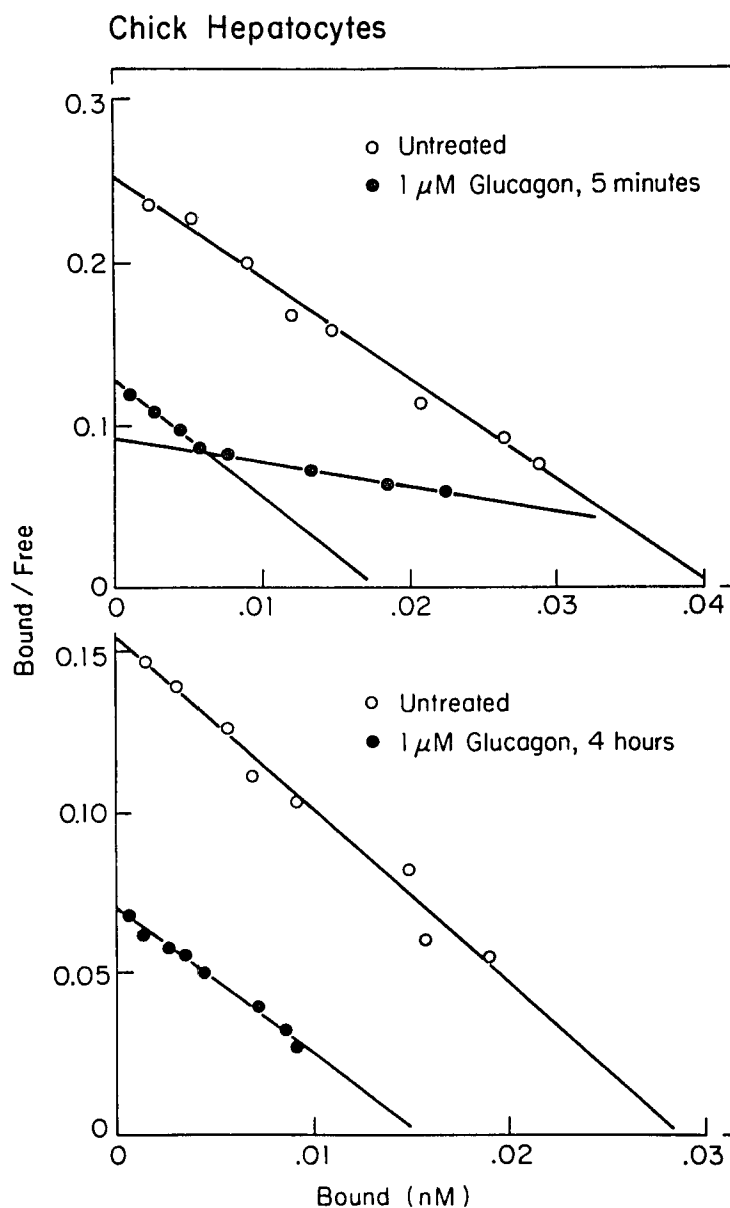


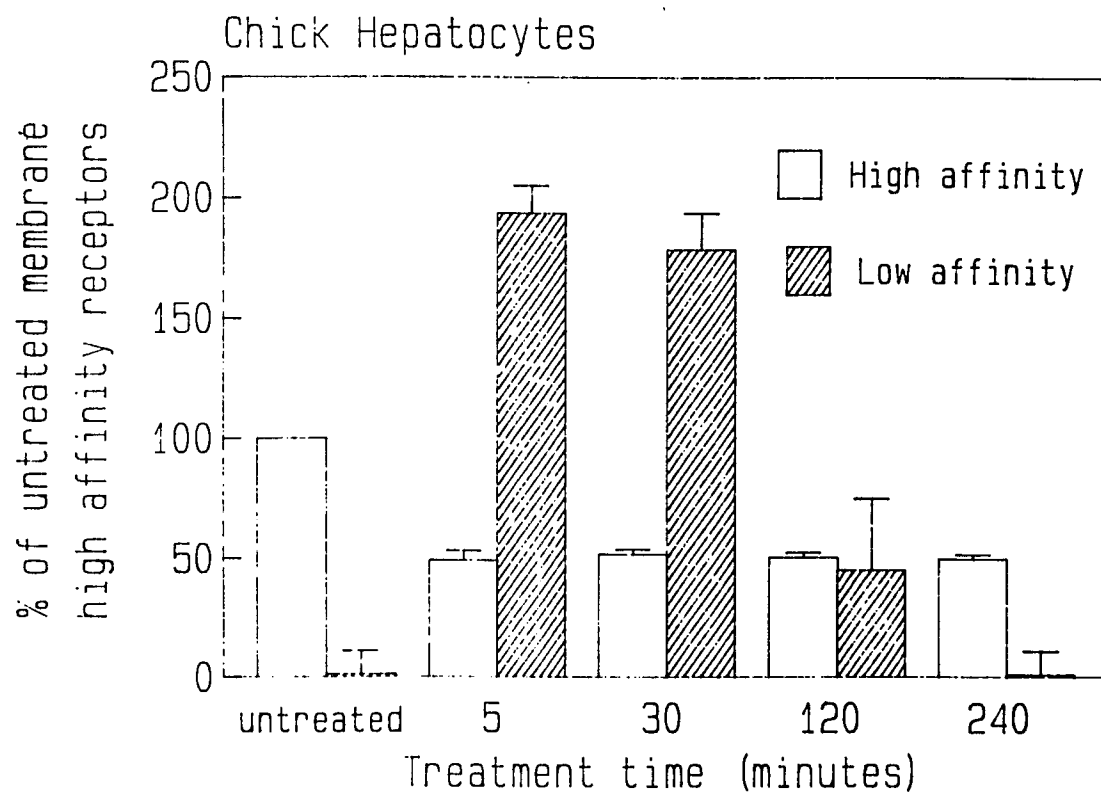
Figure 3-6. Scatchard analysis of [ $^{125}$ I]MIG binding to untreated and glucagon-treated hepatocyte membranes. Hepatocyte membranes were incubated with a range of concentrations, from 0.1 to 10 nM, of [ $^{125}$ I]MIG in the absence and presence of excess glucagon, and binding data shown as Scatchard plots. Top Panel: Hepatocytes were incubated in the absence (open) and presence (closed circles) of 1  $\mu$ M glucagon for 5 min prior to homogenization and membrane preparation. For untreated cells, moniodo-glucagon binding exhibits a single affinity state of 1-2 nM, while short-term glucagon treatment leads to the appearance (or unmasking) of a lower affinity state and a reduction in the apparent number of high affinity binding sites. Bottom Panel: Hepatocytes were incubated in the absence or presence of 1  $\mu$ M glucagon for 4 hours (with media change and hormone readdition after 2 hours) prior to membrane preparation. Both untreated and glucagon-treated cell membranes exhibit a single high affinity binding site of 1-2 nM, although treated membranes appear to have lost 50% of these sites compared to the untreated membranes. Low affinity sites observed following short-term glucagon treatment are not measurable following this longer treatment.



Longer treatment with glucagon, however, produces further changes in the pattern of glucagon binding. In the bottom panel of Figure 3-6, membranes from cells treated with glucagon for 4 hours are once again seen to exhibit only a single high affinity state, but with a 50% reduction in receptor number compared to membranes from untreated cells.

It is difficult to quantitate accurately the low affinity form of the glucagon receptor (and therefore total receptor number) in hepatocyte membranes from Scatchard plots using an agonist receptor probe ( $[^{125}\text{I}]\text{MIG}$ ). Membranes from untreated or 4 hour glucagon-treated cells do not exhibit a measureable low affinity state of the glucagon receptor, although such a low affinity receptor state is likely to be present. Such low affinity receptors would become observable under these assay conditions only when the number of high affinity receptors decreases following glucagon treatment. Therefore, it is not possible to determine unequivocally whether a change in total receptor levels occurs during the early phase of homologous desensitization. Bearing this in mind, the apparent number of low affinity receptors was estimated from Scatchard plots to qualitatively compare alterations in the low affinity receptor following various times of glucagon treatment. Experiments summarized in Figure 3-7 show that after 5 and 30 minutes of treatment with glucagon, both low and high affinity states are seen. After 2 hours of treatment, there is a substantial loss of the low affinity receptors. After 4 hours, the low affinity receptor is once again undetectable. Therefore, short-term treatment with glucagon induces an *apparent* shift in the cellular complement of glucagon receptors from a high affinity to a low affinity state, either by interconversion or by removal of high affinity receptors. In the continued presence of glucagon, however, hepatocytes undergo

Figure 3-7. Apparent levels of high and low affinity glucagon receptors in hepatocyte membranes following glucagon treatment. Hepatocytes were treated for the indicated times with 1  $\mu$ M glucagon prior to homogenization. Membranes were used for [ $^{125}$ I]MIG binding assays at several tracer concentrations, and the resulting Scatchard plots used to estimate the apparent levels of high and low affinity glucagon receptors.



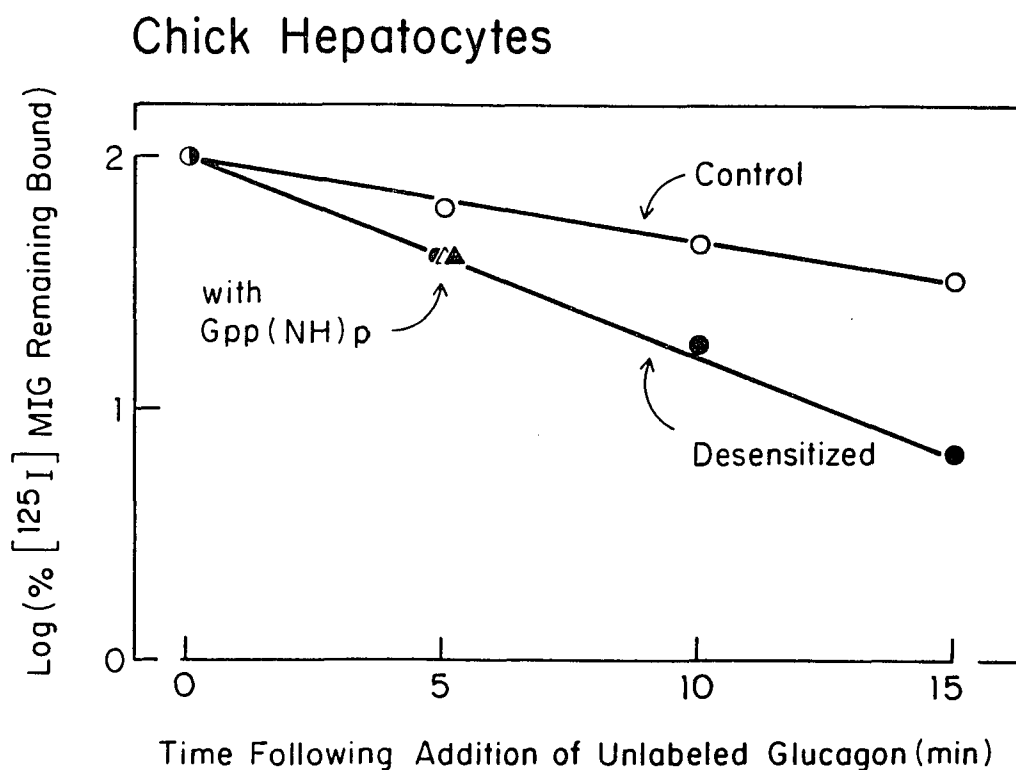
a net loss of the low affinity form of the glucagon receptor, as if the low affinity form of the receptor were preferentially withdrawn from the plasma membranes.

The standard model of receptor loss from the cell surface predicts that the coupled, high-affinity state of the receptor is preferentially removed following agonist exposure, and  $G_s$  appears to be required for this down-regulation process (Mahan *et al*, 1985; Rich & Iyengar, 1989). In the chick hepatocytes, the apparent loss of the low affinity state of the glucagon receptor following prolonged glucagon exposure (Figures 3-6 and 3-7) may result not from direct loss of low affinity (uncoupled) receptors, but from a redistribution of the remaining receptor population following removal of coupled receptors and some recoupling of previously uncoupled receptors.

To examine the role of uncoupling of the glucagon receptor from  $G_s$  in the conversion of receptor from high to low affinity form following glucagon treatment, [ $^{125}$ I]MIG dissociation kinetics in the presence and absence of GppNHp were measured in membranes from untreated and glucagon-treated cells (Figure 3-8). The rate of dissociation of specifically bound [ $^{125}$ I]MIG from glucagon-treated hepatocyte membranes appears to be more rapid than that from untreated cell membranes. Dissociation from the untreated cell membranes is enhanced by addition of guanine nucleotides to a rate similar to that observed in treated cell membranes. Importantly, glucagon treated hepatocyte membranes exhibit the same rapid dissociation rate in the absence or presence of GppNHp. Thus it appears that glucagon receptors in treated membranes exhibit low affinity binding because they are largely uncoupled from  $G_s$ .

Following short-term (5-30 minute) glucagon treatment, crude membranes display apparent two-state [ $^{125}$ I]MIG binding. To determine if this results from interconversion

Figure 3-8. Dissociation of bound [ $^{125}$ I]MIG from membranes from untreated and glucagon-treated hepatocytes. Hepatocytes were incubated in the absence and presence of 1  $\mu$ M glucagon for 5 min prior to homogenization and membrane preparation. 1 nM [ $^{125}$ I]MIG was bound to untreated (open) or glucagon-treated (closed symbols) cell membranes in the absence (circles) or presence (triangles) of 1  $\mu$ M GppNHp. Excess unlabeled glucagon was added, and the binding reaction continued for the indicated times before the label remaining bound was quantified. The log-percent of the maximum bound label is plotted versus time. Glucagon dissociates faster from receptors in membranes from glucagon-treated cells than from untreated, indicating a lower receptor affinity state in the glucagon-treated cells.



of receptors on the cell surface or from the mixing of separate receptor populations on distinct membranes (such as plasma membranes and intracellular vesicles), both untreated and glucagon-treated (for 5 minutes) crude membranes were analyzed by centrifugation to equilibrium in 20-50% sucrose density gradients. Fractions were assayed for [ $^{125}$ I]MIG binding activity and for glucagon- and NaF-stimulated adenylyl cyclase activity, all of which comigrated with a peak at 40% sucrose for both untreated and desensitized cell membranes. Scatchard analysis of [ $^{125}$ I]MIG binding in peak fractions showed that while the untreated cell membranes had only high affinity glucagon receptors, the desensitized cell membranes contained both low and high affinity sites (data not shown). Copurification at a buoyant density indicative of cell surface membranes of both the high and low affinity receptor forms in membranes from treated cells shows that the low affinity receptor does not represent a separate, non-plasma membrane receptor form. On the basis of these observations, we conclude that the low affinity form of the receptor arises from the uncoupling from  $G_s$  of previously coupled, high affinity receptors on the cell surface.

#### Recovery from Homologous Desensitization

The time course of recovery from short-term glucagon desensitization was determined by following the reappearance of [ $^{125}$ I]MIG binding activity and glucagon stimulation of adenylyl cyclase after the removal of the hormone from the media. It was determined to be necessary to rinse cells with glycine buffered saline (pH 4.0) in order to remove glucagon completely from the cell surface and thus allow rapid recovery. In the absence of such a washing procedure, recovery was delayed by the remaining tightly-

bound hormone despite several media changes. Hepatocytes were treated with glucagon for 5 minutes, the cells rinsed in glycine-buffered saline, pH 4.0, and allowed to recover for various times in fresh media prior to preparation of membranes. The recovery of [<sup>125</sup>I]MIG binding activity occurs quite rapidly, with an apparent half-time of 15-20 minutes (Figure 3-9). The glucagon receptors uncoupled by short-term exposure to glucagon appear to recouple to G<sub>s</sub> rapidly following stringent removal of the hormone from the cell surface, as measured by recovery of high affinity binding capacity of the membrane preparation.

Recovery from long-term glucagon treatment, sufficient to induce loss (presumably internalization) of uncoupled glucagon receptors, was also investigated. Full stimulation of adenylyl cyclase by glucagon and full [<sup>125</sup>I]MIG binding recovered after 24, but not 12, hours following removal of glucagon (Figure 3-10). Thus, the homologous component of desensitization develops and recovers in two temporally distinct phases. The uncoupling phase is rapid in its onset and also rapid in its recovery. In contrast, loss of receptors from the cell surface occurs slowly and recovery from receptor loss is also slow.

The ability of the protein synthesis inhibitor cycloheximide to interfere with short-term glucagon-mediated desensitization of adenylyl cyclase was assessed. Hepatocyte membrane glucagon- and NaF-stimulated adenylyl cyclase activities from cell grown for 30 min in the presence of cycloheximide and glucagon were not significantly different from those of hepatocytes grown in the presence of glucagon alone (Table 3-2). Neither the homologous nor the heterologous components of the desensitization response appeared to be affected by cycloheximide treatment, indicating that neither process requires new protein synthesis.

Figure 3-9. Hepatocytes were incubated in the presence of 1  $\mu$ M glucagon for 5 min, rinsed three times with glycine-buffered saline (pH 4.0) and once with media, and allowed to recover in glucagon-free media for the indicated times prior to homogenization. Specific binding activity for 1 nM [ $^{125}$ I]MIG is shown for membranes from the various recovery time points.

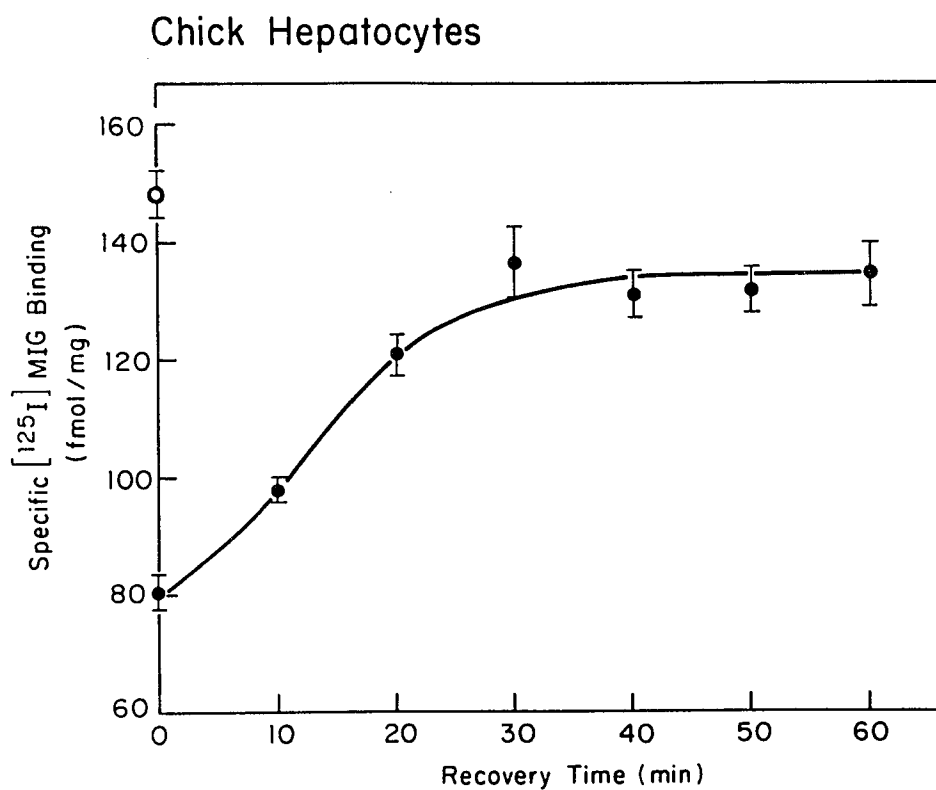
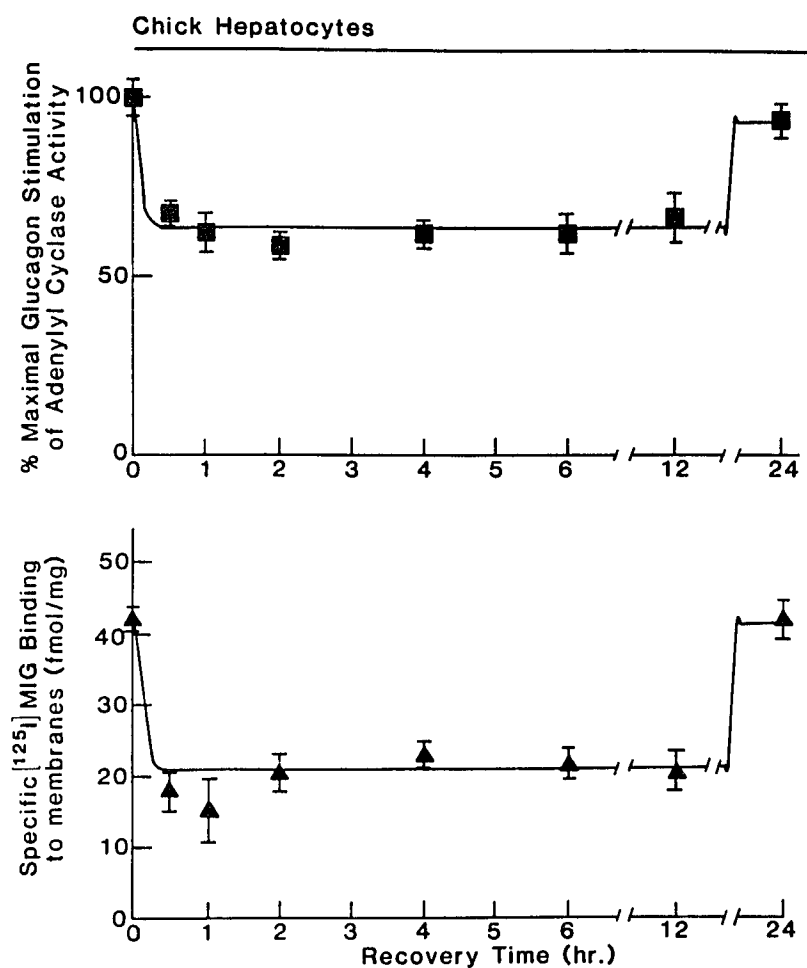


Figure 3-10. Hepatocytes were incubated in the presence of 1  $\mu$ M glucagon for 4 h, with a media change and hormone readdition at 2 h, and then rinsed three times with media and allowed to recover for various times prior to homogenization. Time points indicated are from the beginning of glucagon treatment. *Top panel:* Membranes were assayed for adenylyl cyclase activity stimulated by glucagon and NaF. The ratio of glucagon- to NaF-stimulated adenylyl cyclase activity at each time point is shown as a percentage of the same ratio observed in untreated cell membranes, in order to correct for changing stimulation due to concurrent heterologous desensitization. *Bottom panel:* Membranes were assayed for specific [ $^{125}$ I]MIG binding activity at 1 nM tracer, and activity is plotted versus recovery time.



Hepatocytes treated with glucagon for 4 hours were allowed to recover overnight in the presence and absence of the protein synthesis inhibitor cycloheximide in order to assess the role of protein synthesis in recovery of lost glucagon receptors. As seen in Table 3-3, treatment with 0.5  $\mu\text{g/ml}$  cycloheximide overnight is sufficient to inhibit the incorporation of [ $^3\text{H}$ ]-leucine into TCA-precipitable protein by 93%, without significant effects on cell viability. In the presence of 0.5  $\mu\text{g/ml}$  cycloheximide, stimulation of adenylyl cyclase by glucagon and glucagon receptor binding activity recover substantially although only partially, to a level between that of desensitized and recovered (in the absence of cycloheximide) hepatocyte membranes (Table 3-4). It should be noted, however, that overnight cycloheximide treatment significantly reduced both basal and stimulated adenylyl cyclase activities in hepatocytes. Glucagon binding activity recovers following glucagon treatment to the same level in the presence of cycloheximide as is measured in cycloheximide-treated cells which have never seen the hormone (but is less than that observed in those cells that have never seen either compound). The decreased [ $^{125}\text{I}$ ]MIG binding activity observed following overnight treatment with cycloheximide is presumably due to background turnover of the glucagon receptor protein, since only new synthesis is blocked by cycloheximide while receptor destruction should continue at its normal pace. Nevertheless, the observed recovery indicates that the glucagon receptors lost from the cell surface in response to glucagon treatment are not entirely degraded within 24 hours. Glucagon receptors internalized following prolonged (2-4 h) treatment of hepatocytes with glucagon appear to be slowly recycled to the cell surface, although the low recovery observed following cycloheximide treatment indicates that many of the recovered receptors may actually be newly synthesized proteins.

Table 3-2. Effect of cycloheximide on desensitization following short-term glucagon treatment. Hepatocytes were incubated in the absence or presence of 1  $\mu$ M glucagon, or in the presence of glucagon and 0.5  $\mu$ g/ml cycloheximide, for 30 min prior to cell homogenization. Membrane adenylyl cyclase activity for one experiment is shown as mean  $\pm$  SD for triplicate determinations.

| cell treatment           | adenylyl cyclase activity<br>(pmol/min/mg)<br>in the presence of |                 |
|--------------------------|------------------------------------------------------------------|-----------------|
|                          | glucagon                                                         | NaF             |
|                          | .....                                                            |                 |
| no treatment             | 3.03 $\pm$ 0.37                                                  | 3.84 $\pm$ 0.18 |
| glucagon                 | 1.28 $\pm$ 0.11                                                  | 3.15 $\pm$ 0.15 |
| glucagon + cycloheximide | 1.34 $\pm$ 0.15                                                  | 3.02 $\pm$ 0.16 |

Table 3-3. Effect of cycloheximide treatment on hepatocyte protein synthesis and cell viability. Hepatocytes were grown in the presence of 0.5  $\mu\text{g/ml}$  cycloheximide for 18 h. Protein synthesis was monitored by incorporation of [ $^3\text{H}$ ]-leucine into TCA-precipitable counts. Cells were grown with 0.1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-leucine in leucine-free media in the presence and absence of cycloheximide. Cells were lysed with 10% TCA and precipitated at 10,000xg for 10 min. Pellets were washed with 10% TCA and resuspended in 1 N NaOH. Radioactivity remaining was quantified by scintillation counting of triplicate aliquots, and pellet protein was determined by the method of Lowry *et al* (1951). Viability was determined by trypan blue exclusion of cells from parallel cultures.

| Cell treatment | [ $^3\text{H}$ ]-leucine<br>incorporation<br>(cpm/mg) | % viability |
|----------------|-------------------------------------------------------|-------------|
| none           | 54,961 $\pm$ 426                                      | 94          |
| cycloheximide  | 3,691 $\pm$ 73                                        | 92          |

Table 3-4. Effect of cycloheximide on receptor recovery following prolonged glucagon treatment. Hepatocytes were grown in the presence of 1  $\mu$ M glucagon for 4 h, with a media change and hormone readdition at 2 h, and rinsed three times with glycine-buffered saline (pH 4.0) and then with media (pretreatment). Cells were then incubated for 18 h in hormone-free media in the absence and presence of 0.5  $\mu$ g/ml cycloheximide prior to homogenization (recovery). Membrane adenylyl cyclase activity stimulated by glucagon and NaF was determined, as was [ $^{125}$ I]MIG binding activity at 1 nM tracer. Glucagon-stimulated adenylyl cyclase activity was divided by NaF-stimulated activity of the same membranes and is shown as a percentage of the glucagon/NaF-stimulation ratio measured in untreated cell membranes, to correct for decreased adenylyl cyclase activity observed following cycloheximide treatment alone. Cells grown in the presence of 0.5  $\mu$ g/ml cycloheximide exhibited a 50% reduction in absolute adenylyl cyclase activities, for both with and without glucagon treatment groups.

| Cell treatment   |               | glucagon stimulation                    | [ $^{125}$ I]MIG              |
|------------------|---------------|-----------------------------------------|-------------------------------|
| 4 h pretreatment | 18 h recovery | of adenylyl cyclase<br>(% NaF activity) | binding activity<br>(fmol/mg) |
| no addition      | no addition   | 100.0                                   | 148.2 $\pm$ 7.3               |
| glucagon, 4 h    | NONE          | 38.2                                    | 69.9 $\pm$ 4.6                |
| glucagon, 4 h    | no addition   | 83.2                                    | 151.4 $\pm$ 8.3               |
| no addition      | cycloheximide | 97.3                                    | 101.6 $\pm$ 3.8               |
| glucagon, 4 h    | cycloheximide | 66.5                                    | 105.1 $\pm$ 4.1               |

## Summary

Treatment of chick hepatocytes in primary culture with glucagon for short periods induces alterations in the glucagon-stimulated adenylyl cyclase system which can be observed in subsequently prepared cell membranes. Glucagon treatment appears to produce both glucagon receptor-specific (homologous) and non-specific (heterologous) alterations in the hepatocytes. A substantial (60%) loss of glucagon-stimulated adenylyl cyclase activity evident within 5 min of glucagon exposure has been used to measure the homologous desensitization, while a smaller (20%) loss of NaF-stimulated adenylyl cyclase activity evident within 15-20 min following glucagon exposure was used to measure the heterologous desensitization.

The homologous desensitization of glucagon-stimulated adenylyl cyclase activity is concomitant with the apparent loss of glucagon binding activity in cell membranes. Scatchard analysis of [ $^{125}$ I]MIG binding at varying concentrations indicates that the apparent loss of binding at fixed tracer is due to two separate events. An initial loss of a high affinity binding sites with the unmasking of low affinity sites is observed within 5 min, while prolonged treatment with glucagon (2-4 h) induces a further loss of the low affinity sites as well. At shorter treatment times (5 min), glucagon receptors in glucagon treated cell membranes exhibit a more rapid rate of [ $^{125}$ I]MIG dissociation than do those in untreated cell membranes. In untreated cell membranes, the dissociation rate can be increased by addition of guanine nucleotide analogs, indicating that the receptors were coupled to  $G_s$ . In the glucagon-treated hepatocyte membranes, however, GppNHp is unable to further increase the already rapid dissociation rate, indicating that these low affinity receptors are substantially uncoupled from  $G_s$ . Taken together, glucagon

treatment induces rapid uncoupling of glucagon receptors from  $G_s$ , resulting in reduced affinity for agonists, and later internalization of receptors from the cell surface membrane.

Removal of glucagon from hepatocytes following a short treatment (sufficient to induce the rapid uncoupling of the receptor from  $G_s$ ) allows the rapid (15-30 min) recovery of both glucagon binding activity and adenylyl cyclase stimulation by glucagon. However, this recovery is dependent on a highly stringent rinse of the cells with low pH to remove glucagon completely from the cell surface. Simply changing the media is insufficient to allow recovery of activity, indicating that rather little constant stimulation is required to maintain the desensitization. After longer glucagon treatments (sufficient to induce the slower removal of glucagon receptors from the cell surface), the recovery of glucagon binding and glucagon-stimulated adenylyl cyclase activity also requires longer periods (12-16 h). Internalized receptors appear to be recycled to the cell surface to some extent. The mechanisms for targeting the glucagon receptors for internalization or for return to the cell surface are presently unknown.

The rapidity of the first phase of homologous desensitization (glucagon receptor uncoupling) in both onset and recovery imply that a rapid and easily reversible mechanism must be responsible. The phosphorylation of the glucagon-bound receptor protein by a receptor kinase and the subsequent binding of an arrestin to the phosphorylated receptor appear a likely mechanism. The direct demonstration of glucagon receptor phosphorylation by a BARK-like kinase is a difficult task, since there are no efficient means for purifying the receptor protein at present. Monoclonal antibodies have been prepared recently against the rat liver glucagon receptor, but none crossreact with chick liver receptor (Iwanij & Vincent, 1991). I had attempted to utilize

a immune-sandwich technique to immunoprecipitate the glucagon receptor using anti-glucagon antisera and glucagon-crosslinked receptors. The receptor crosslinked to [<sup>125</sup>I]MIG can be specifically immunoprecipitated by this technique (RTP and RI, unpublished observations), but the low efficiency of the crosslinking and the precipitation steps make it unsuitable for reproducibly demonstrating receptor phosphorylation.

In the extensively characterized  $\beta$ -adrenergic system, regulation of the receptor protein in homologous desensitization appears to occur in three phases: receptor-G<sub>s</sub> uncoupling, receptor sequestration, and receptor down-regulation (Sibley & Lefkowitz, 1985). The cloning of the cDNAs and genes for many adrenergic receptor subtypes is allowing a great expansion of the knowledge of the mechanisms responsible for each of these processes, through expression and mutation (Clark *et al*, 1988; Liggett *et al*, 1989; Hausdorff *et al*, 1989). However, no discrete receptor sequestration and down-regulation steps have been observed for the glucagon receptor due to the lack of appropriate ligand probes. The receptor internalization observed using the agonist probe [<sup>125</sup>I]MIG may represent a combination of both processes. Indeed, such a distinction could probably be observed in the hepatocytes by analysis of recovery from long-term glucagon treatment. I measured recovery of internalized glucagon receptors following 4h treatment with glucagon (Figure 3-10), which is sufficient time to remove the receptors from the cell surface (Figure 3-7). At this time, recovery is a very slow process, and full recovery appears to require some new protein synthesis (Table 3-4). This treatment, then, appears to induce receptor down-regulation rather than sequestration. Treatment for shorter times may allow a more rapid recovery, indicating that receptors are sequestered rather than down-regulated. Mechanistically, the distinction is not entirely irrelevant, since analysis

of receptor loss and recovery for  $\beta$ -adrenergic receptors indicates that sequestration and down-regulation of receptors are not sequential events in a single pathway but independent alterations (Rich & Iyengar, 1989).

Glucagon-induced desensitization of adenylyl cyclase has been studied by other workers in several cell systems. Cultured rat hepatocytes have been reported to undergo both homologous and heterologous desensitizations (Gurr & Ruh, 1980; Noda *et al*, 1984; Refsnes *et al*, 1989), while freshly isolated rat hepatocytes (Santos & Blasquez, 1982; Heyworth & Houslay, 1983) and rat liver slices (DeRobertis & Craven, 1976) were reported to undergo only homologous desensitization. Cultured canine kidney MDCK cells (Rich *et al*, 1984) and primary rat Sertoli cells (Attramadal *et al*, 1988) have also been reported to undergo both homologous and heterologous desensitizations.

In these studies, homologous desensitization appeared to be due to the apparent uncoupling and internalization of the glucagon receptor. Heterologous desensitization, on the other hand, was thought to be due to cAMP-dependent phosphorylation of "regulatory factors" (Gurr & Ruh, 1980), functional modification but not loss of  $G_s$  (Noda *et al*, 1984), apparent increases in  $G_i$  protein with no change in  $G_s$  activity (Rich *et al*, 1984), cAMP-dependent functional alteration of  $G_s$  (Attramadal *et al*, 1988), and cAMP-independent functional alteration of  $G_s$  (Refsnes *et al*, 1989). Further, in most of these studies, this heterologous effect was much slower (1-4 h) than that observed in chick hepatocytes (15-20 min, Figure 3-4). Due to this clear lack of consensus as to the mechanisms responsible for heterologous desensitization led to further studies to define the mechanisms responsible for the rapid heterologous desensitization observed in chick hepatocytes.

## CHAPTER 4

### HETEROLOGOUS DESENSITIZATION: REGULATION AT THE LEVEL OF G PROTEINS

The role of glucagon receptor regulation in homologous desensitization of the chick hepatocyte adenylyl cyclase system appears essentially similar to that of the  $\beta$ -adrenergic receptor system (Sibley & Lefkowitz, 1985). Whether the observed glucagon receptor uncoupling from  $G_s$  is associated with agonist-dependent receptor phosphorylation by BARK or a BARK-like protein kinase, or what mechanisms are responsible for receptor down-regulation, remain to be determined when suitable tools are developed to purify or clone the glucagon receptor. In addition to this homologous-type desensitization, however, chick hepatocytes also undergo heterologous desensitization of adenylyl cyclase following treatment with glucagon. This heterologous desensitization is associated with reductions in both hormonal- and NaF-stimulated adenylyl cyclase activities (Table 3-1), and is characterized by a rapid (15-20 min) onset (Figure 3-4).

Heterologous desensitization of adenylyl cyclase has been best characterized for the  $\beta$ -adrenergic receptor system. Several distinct types of heterologous desensitizations have been reported, but few have been extensively characterized. One form is only observed in intact cells, and may be due to hormonal modulation of cAMP phosphodiesterase activity (reviewed in Harden, 1983; Clark, 1986). Other types of

desensitization survive disruption of the cell, and appear to result from modulation of receptors and G proteins.

In many cell types, a form of heterologous desensitization results from the cAMP-dependent phosphorylation of hormone receptors, irrespective of agonist occupancy state, by the cAMP-dependent protein kinase (Sibley *et al*, 1984b; Benovic *et al*, 1985; Clark *et al*, 1988; Hausdorff *et al*, 1989). This modification somehow results in the functional uncoupling of the receptors from  $G_s$ . However, this type of desensitization has no effect on  $G_s$  or on NaF-stimulated adenylyl cyclase activity.

A hormone-induced reduction in  $G_s$ -dependent adenylyl cyclase activity has been observed in several systems (Kassis & Fishman, 1982; Garrity *et al*, 1983; Kirchick *et al*, 1983). This loss affects adenylyl cyclase stimulation through all  $G_s$ -coupled receptor types on the cell surface, and can be assayed following detergent extraction of  $G_s$  from the desensitized cell membranes and its reconstitution with adenylyl cyclase in membranes from *cyc*<sup>-</sup> S49 lymphoma cells (Ross & Gilman, 1978a,b), which lack native  $\alpha_s$  mRNA or protein (Harris *et al*, 1985). Such reductions in  $G_s$  activity have most-often been ascribed to some covalent modification of the  $G_s$  protein, often by phosphorylation. However,  $\alpha_s$  has no predicted protein kinase A sites (Bray *et al*, 1986), and other protein kinases which might be stimulated to phosphorylate  $G_s$  in response to agonist are not currently known. Heterologous desensitization through an adenylyl cyclase *inhibitory* receptor has been reported to lead to elevated  $G_s$  activity (Parsons & Stiles, 1987). Modulations of G protein subunit levels have also been reported following desensitization (Garrity *et al*, 1983; Rich *et al*, 1984; Reithmann *et al*, 1991).

The mechanisms responsible for heterologous desensitization of the adenylyl cyclase system are much less well understood than those resulting in homologous desensitization. The rapid onset time for glucagon-induced heterologous desensitization in chick hepatocytes is in contrast with slower glucagon-induced heterologous desensitization in other systems (Rich *et al*, 1984; Noda *et al*, 1984; Attramadal *et al*, 1988). Therefore, I set out to further characterize the locus of glucagon-induced heterologous desensitization in the chick hepatocyte system.

#### Induction of Heterologous Desensitization by Other Hormones

Treatment of chick hepatocytes with 1  $\mu$ M glucagon, 10  $\mu$ M PGE<sub>1</sub>, 50  $\mu$ M (-) isoproterenol, or 2  $\mu$ M VIP for 30 minutes prior to preparation of membranes leads to reductions in adenylyl cyclase stimulation by NaF and by glucagon (Table 4-1). The extent of loss of both glucagon- and NaF-stimulation of adenylyl cyclase is comparable following treatment with each of these hormones (except glucagon), and of the same magnitude as the loss of NaF stimulated adenylyl cyclase activity following glucagon treatment. This is in contrast with the much larger loss of glucagon stimulation following glucagon treatment. The loss of glucagon stimulation following glucagon treatment, after taking into account the loss of NaF (G<sub>s</sub>-dependent) stimulation, thus appears to be glucagon-specific, and represents the homologous component of the desensitization. Measurement of adenylyl cyclase activity stimulated by each of these other hormones following treatment of hepatocytes also shows a greater reduction in activity compared to NaF, indicating that each of these hormones may induce a homologous desensitization of its own receptors (data not shown). Homologous desensitization induced by these

other hormones has not been further studied.  $\text{PGE}_1$ , isoproterenol, or VIP treatments have no effect on [ $^{125}\text{I}$ ]MIG binding activity (Table 4-1), indicating that the loss of glucagon binding activity due to glucagon receptor modulation is also glucagon-specific. Treatment of cells with hormones which do not stimulate hepatocyte membrane adenylyl cyclase, such as angiotensin II or vasopressin, is without effect on adenylyl cyclase activity or [ $^{125}\text{I}$ ]MIG binding activity. A common heterologous desensitization induced by hormones which couple to  $G_s$  and stimulate adenylyl cyclase thus appears to occur in chick hepatocytes, in addition to the glucagon-specific homologous desensitization characterized in the last chapter.

#### Induction of Heterologous Desensitization by Direct Activation of Protein Kinases

To test for the cAMP-dependence of the heterologous desensitization, hepatocytes were treated with 10  $\mu\text{M}$  forskolin or with 10  $\mu\text{M}$  8-Br-cAMP for 30 minutes. Adenylyl cyclase activities of membranes prepared from treated cells are given in Table 4-2 for one such experiment. Forskolin treatment had no consistent effect on adenylyl cyclase activities; in some experiments activity decreased, while in others it was increased up to 2-fold. This may be due in part to incomplete removal of the hydrophobic forskolin from the membranes during preparation, since forskolin is known to enhance hormonal stimulation of adenylyl cyclase at concentrations lower than those required for independent stimulation of adenylyl cyclase (Seamon & Daly, 1981b). 8-Br-cAMP treatment consistently induced a small reduction in both glucagon- and NaF-stimulated activity, but the magnitude of reduction was somewhat less than that seen following treatment with glucagon. Additionally, neither forskolin nor 8-Br-cAMP treatment affected

Table 4-1. Effects of hormonal treatment of hepatocytes on adenylyl cyclase activities and [ $^{125}$ I]MIG binding activity. Hepatocytes were grown for 30 min in the presence of the indicated concentrations of various hormones prior to homogenization. Membrane adenylyl cyclase activity stimulated by glucagon and by NaF, and [ $^{125}$ I]MIG binding activity at 1 nM tracer, were determined for each cell treatment group. Values obtained are shown as mean  $\pm$  SD of triplicate determinations.

| cell treatment                | adenylyl cyclase activity<br>(pmol/min/mg)<br>in the presence of |                 | [ $^{125}$ I]MIG<br>binding activity<br>(fmol/mg) |
|-------------------------------|------------------------------------------------------------------|-----------------|---------------------------------------------------|
|                               | glucagon                                                         | NaF             |                                                   |
| no treatment                  | 3.36 $\pm$ 0.12                                                  | 3.27 $\pm$ 0.06 | 84.3 $\pm$ 3.4                                    |
| glucagon, 1 $\mu$ M           | 1.61 $\pm$ 0.08                                                  | 2.88 $\pm$ 0.08 | 29.7 $\pm$ 1.0                                    |
| (-)-iso, 50 $\mu$ M           | 2.81 $\pm$ 0.18                                                  | 2.77 $\pm$ 0.16 | 79.3 $\pm$ 2.4                                    |
| PGE <sub>1</sub> , 10 $\mu$ M | 2.83 $\pm$ 0.07                                                  | 2.80 $\pm$ 0.21 | 81.5 $\pm$ 4.5                                    |
| VIP, 2 $\mu$ M                | 2.76 $\pm$ 0.04                                                  | 2.72 $\pm$ 0.13 | 78.7 $\pm$ 3.2                                    |
| angiotensin II, 10 $\mu$ M    | 3.33 $\pm$ 0.16                                                  | 3.24 $\pm$ 0.19 | 81.1 $\pm$ 2.4                                    |
| vasopresin, 10 $\mu$ M        | 3.57 $\pm$ 0.15                                                  | 3.45 $\pm$ 0.11 | 83.8 $\pm$ 3.7                                    |

Table 4-2. Effects of cAMP-elevating agent treatment of hepatocytes on adenylyl cyclase and [ $^{125}$ I]MIG binding activities. Hepatocytes were incubated for 30 min in the presence of the indicated concentrations of cAMP-elevating agents prior to homogenization. Membranes were assayed for adenylyl cyclase and [ $^{125}$ I]MIG (1 nM) binding activities, and the results for one experiment are shown as mean  $\pm$  SD of triplicate determinations.

| cell treatment        | adenylyl cyclase activity<br>(pmol/min/mg)<br>in the presence of |                  | [ $^{125}$ I]MIG<br>binding activity<br>(fmol/mg) |
|-----------------------|------------------------------------------------------------------|------------------|---------------------------------------------------|
|                       | glucagon                                                         | NaF              |                                                   |
| no treatment          | 9.21 $\pm$ 0.87                                                  | 15.74 $\pm$ 0.61 | 54.6 $\pm$ 2.6                                    |
| glucagon, 1 $\mu$ M   | 3.05 $\pm$ 0.47                                                  | 8.96 $\pm$ 1.18  | 33.5 $\pm$ 2.3                                    |
| forskolin, 10 $\mu$ M | 9.87 $\pm$ 0.41                                                  | 16.88 $\pm$ 1.05 | 57.1 $\pm$ 2.9                                    |
| 8-Br-cAMP, 10 $\mu$ M | 7.88 $\pm$ 1.06                                                  | 13.31 $\pm$ 0.62 | 55.7 $\pm$ 2.2                                    |

the binding capacity for [ $^{125}$ I]MIG. Treatment with 8-Br-cAMP in higher doses or for longer periods does not produce further reduction in adenylyl cyclase activity (data not shown). Therefore, heterologous desensitization does not appear to be mediated exclusively by increases in cellular cAMP levels, although it appears to play a role.

Treatment of rat hepatocytes with TPA, a protein kinase C-activating phorbol ester, or diacylglycerol analogs has been reported to result in a state resembling desensitization (Heyworth *et al*, 1984b; 1985; Newlands & Houslay, 1991). As shown in Table 4-3, treatment of chick hepatocytes with TPA resulted in no measurable effect on glucagon-stimulated adenylyl cyclase activity, and only slightly enhanced NaF-stimulated adenylyl cyclase activity at quite high doses. Further, TPA and glucagon cotreatment had no effects not observed with glucagon alone. Therefore, TPA has no effect on the development of homologous or heterologous desensitization, nor on GTP regulation of glucagon ([ $^{125}$ I]MIG) binding (data not shown).

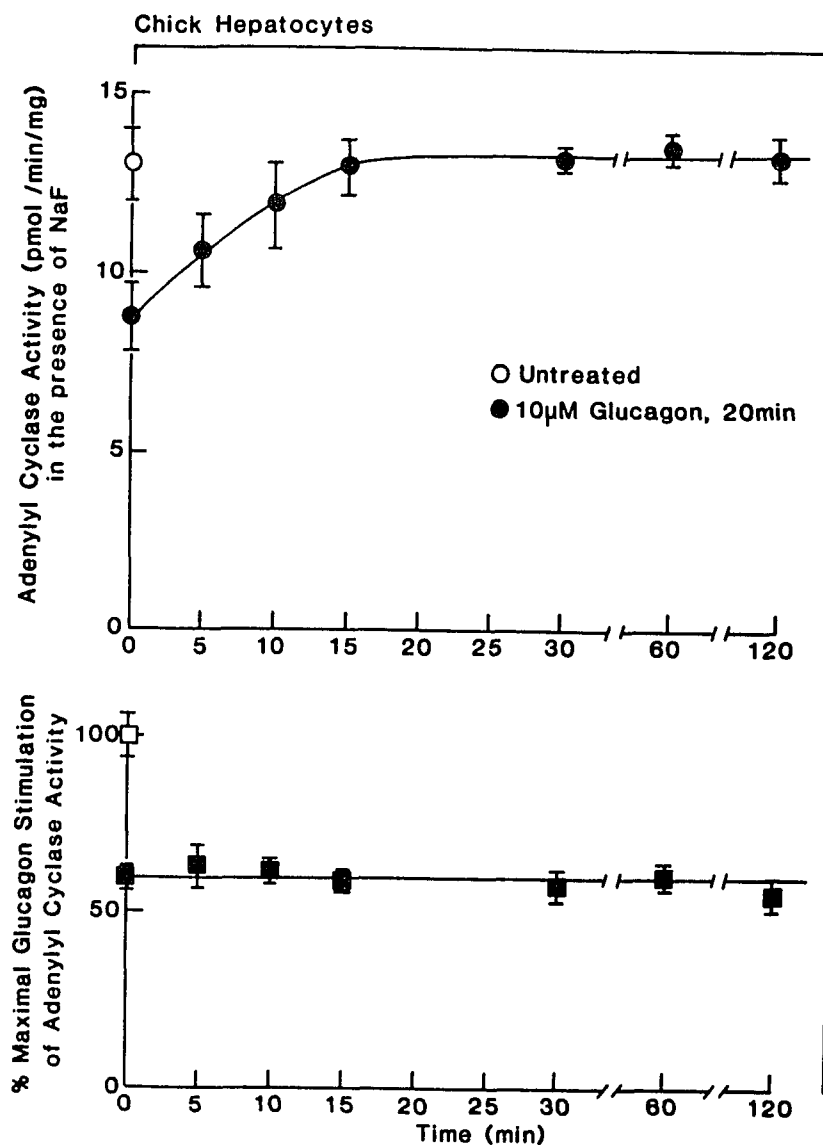
#### Recovery from Heterologous Desensitization

Hepatocytes were desensitized by treatment with 10  $\mu$ M glucagon for 20 minutes, the cells were then washed 3 times with fresh media, and allowed to recover in glucagon-free media for various times. Despite the high concentration of glucagon during treatment, and evidence that all of the glucagon is not removed during the recovery phase (see below), the NaF-stimulated adenylyl cyclase activity recovers quite rapidly (Figure 4-1). Under these conditions for the removal of glucagon, there is no measurable recovery of glucagon-stimulated adenylyl cyclase activity nor of specific [ $^{125}$ I]MIG binding activity (data not shown) within 2 hours after removal of glucagon. Removal of

Table 4-3. Effects of phorbol ester TPA treatment of hepatocytes on membrane adenylyl cyclase activity. Hepatocytes were incubated for 30 min in the presence of various concentrations of TPA (or TPA plus 1  $\mu$ M glucagon) prior to homogenization. TPA was dissolved and diluted in DMSO, so that all cells received a final concentration of 1% DMSO. Membranes were assayed for adenylyl cyclase activity stimulated by NaF or glucagon, and the results for one experiment are shown as mean  $\pm$  SD of triplicate determinations.

| cell treatment                            | adenylyl cyclase activity<br>(pmol/min/mg)<br>in the presence of |                 |
|-------------------------------------------|------------------------------------------------------------------|-----------------|
|                                           | glucagon                                                         | NaF             |
| no treatment                              | 3.24 $\pm$ 0.18                                                  | 4.05 $\pm$ 0.12 |
| 1 $\mu$ M glucagon                        | 1.40 $\pm$ 0.04                                                  | 3.29 $\pm$ 0.18 |
| 1 ng/ml TPA                               | 3.38 $\pm$ 0.05                                                  | 4.27 $\pm$ 0.21 |
| 10 ng/ml TPA                              | 3.17 $\pm$ 0.17                                                  | 3.96 $\pm$ 0.15 |
| 100 ng/ml TPA                             | 3.44 $\pm$ 0.05                                                  | 4.42 $\pm$ 0.20 |
| 1 $\mu$ g/ml TPA                          | 3.34 $\pm$ 0.09                                                  | 4.40 $\pm$ 0.13 |
| 10 $\mu$ g/ml TPA                         | 3.34 $\pm$ 0.14                                                  | 4.36 $\pm$ 0.05 |
| 10 $\mu$ g/ml TPA +<br>1 $\mu$ M glucagon | 1.40 $\pm$ 0.11                                                  | 3.55 $\pm$ 0.12 |

Figure 4-1. Recovery from short-term heterologous desensitization. Hepatocytes were treated with 10  $\mu$ M glucagon for 20 min, rinsed with media, and incubated in fresh media. No low pH wash step was used to remove all bound glucagon. Cells were allowed to recover for the indicated times prior to homogenization. Panel A: Membrane adenylyl cyclase activity stimulated by NaF. Panel B: Adenylyl cyclase activity stimulated by glucagon, shown as a percentage of NaF stimulated activity at the same time point.



glucagon from the cells is incomplete in the absence of rinsing with glycine buffered saline (pH 4.0) as evidenced by the lack of receptor recovery (see Figure 3-9). Additionally, elevated adenylyl cyclase activity in the absence of added glucagon in treated cell membranes indicates the presence of remaining hormone following rinsing and recovery of cells and subsequent preparation of membranes.

The experiment in Figure 4-2 was designed to demonstrate the rapidity with which heterologous desensitization can be reversed. Hepatocytes were exposed to 1  $\mu$ M glucagon for two minutes, and glucagon was removed by rinsing the cells in phosphate-buffered saline, pH 7.3, prior to changing to fresh media. Under these conditions, both homologous and heterologous desensitization occur, as measured by glucagon- and NaF-stimulation of adenylyl cyclase, respectively. The transient nature of the heterologous desensitization is highlighted by its rapid reversal following withdrawal of hormone under conditions where homologous desensitization is maintained (there was no low pH rinse). Treatment of hepatocytes with 5  $\mu$ g/ml cycloheximide has no effect on the rapid development of (Table 3-4) or recovery from (Table 4-4) this glucagon-induced heterologous desensitization.

The rapid reversal of this heterologous phase of desensitization upon lowering of glucagon demonstrates that recovery from heterologous and homologous desensitization are distinct events, differing in their sensitivity to the concentration of glucagon required to suppress recovery. The relatively rapid onset and recovery of the heterologous desensitization and the fact that stable desensitization can be observed in membranes prepared from treated cells indicates that some form of reversible modification of the adenylyl cyclase system must be responsible.

Figure 4-2. Rapid onset and recovery from glucagon-induced heterologous desensitization. Hepatocytes were incubated in the presence of 1  $\mu$ M glucagon for 2 min, rinsed in PBS (pH 7.3), and allowed to continue incubation in fresh media for the indicated times prior to homogenization. Adenylyl cyclase activity stimulated by NaF (closed) and glucagon (open circles) is shown versus time following removal of glucagon.

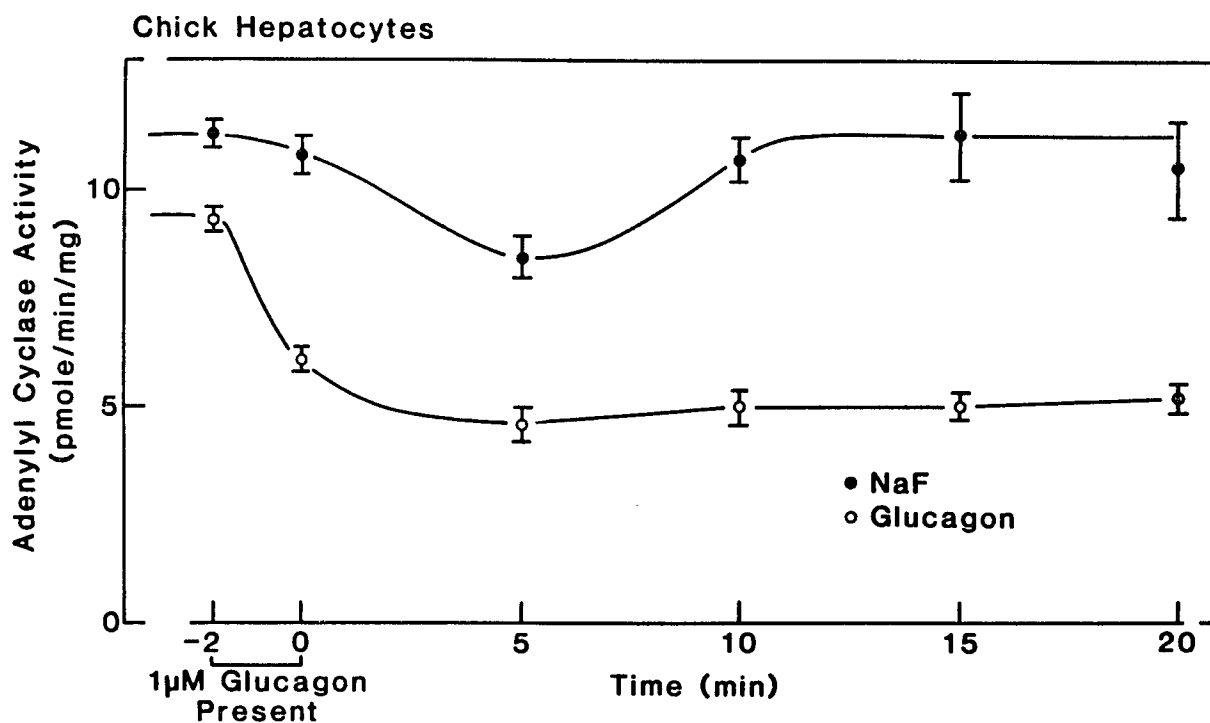


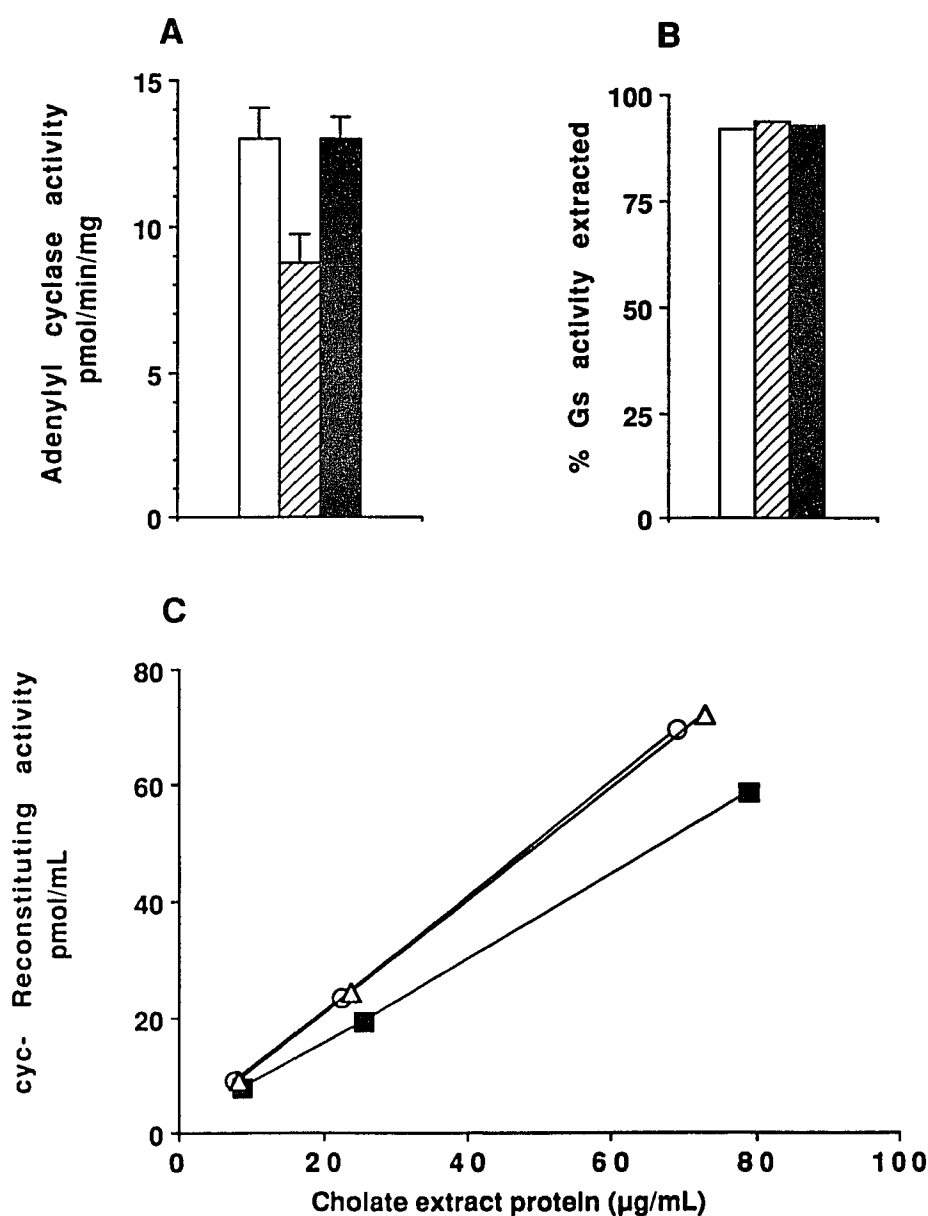
Table 4-4. Effects of cycloheximide on recovery from short-term glucagon treatment. Hepatocytes were incubated for 30 min in the absence or presence of 1  $\mu$ M glucagon or glucagon. Cells treated with glucagon were rinsed with media and allowed to recover for 30 min in the absence of hormone, and in the absence or presence of 0.5  $\mu$ g/ml cycloheximide, prior to homogenization. Membrane adenylyl cyclase activity stimulated by glucagon or NaF was determined for each treatment group.

| cell treatment                            | adenylyl cyclase activity<br>(pmol/min/mg)<br>in the presence of |                 |
|-------------------------------------------|------------------------------------------------------------------|-----------------|
|                                           | glucagon                                                         | NaF             |
| no treatment                              | 8.56 $\pm$ 0.37                                                  | 9.88 $\pm$ 0.28 |
| glucagon 30 min                           | 3.84 $\pm$ 0.25                                                  | 8.12 $\pm$ 0.23 |
| glucagon 30 min +<br>recovery 30 min      | 4.43 $\pm$ 0.27                                                  | 9.74 $\pm$ 0.19 |
| glucagon 30 min +<br>cycloheximide 30 min | 4.63 $\pm$ 0.30                                                  | 9.97 $\pm$ 0.26 |

### Regulation of $G_s$ activity

In order to explore the mechanisms allowing such rapid onset of and recovery from heterologous desensitization, the  $G_s$  activity of cholate extracts prepared from membranes of hepatocytes treated with glucagon and then allowed to recover for short periods of time was measured by reconstitution with adenylyl cyclase present in membranes from *cyc<sup>-</sup>* S49 cells, which lacks  $G_s$  (Harris *et al*, 1985). In Figure 4-3, panel A, glucagon treatment for 30 min reduces NaF-stimulated adenylyl cyclase activity in hepatocyte membranes by 22%; upon removal of hormone by changing the media, and following a 30 min incubation in hormone-free media, adenylyl cyclase activity is restored to control levels. In panel C, glucagon-treated membranes extracted with 1% cholate and reconstituted with *cyc<sup>-</sup>* S49 cell membranes exhibit 20% less NaF-stimulated  $G_s$  activity than those from untreated hepatocytes. Cholate extracts prepared from membranes from glucagon-treated hepatocytes allowed to recover for 30 min prior to homogenization are essentially indistinguishable from those from untreated cell membranes. In panel B, the ratio of  $G_s$  activity in the 100,000xg cholate extract to that in the unfractionated cholate suspensions was determined, and the similarity among the samples indicates that the differences seen in panel C are not due to artifacts of variable  $G_s$  extraction in the different treatment groups. The recovery of this lowered  $G_s$  activity within 30 min of the removal of glucagon indicates that glucagon regulation of the amount of  $G_s$  activity in the membrane is acute and reversible. Similar reductions in *cyc<sup>-</sup>* S49 cell membrane adenylyl cyclase reconstituting activity were also observed in detergent extracts of membranes from hepatocyte treated with glucagon for 2 h (data not shown).

Figure 4-3. Measurement of  $G_s$  activity in hepatocyte membrane extracts by reconstitution of S49 *cyc* cell membrane adenylyl cyclase. Hepatocytes were incubated for 30 min in the presence of 1  $\mu$ M glucagon, rinsed, and allowed to recover in fresh media an additional 30 min prior to homogenization. **Panel A:** NaF-stimulated adenylyl cyclase activity of untreated (open), glucagon-treated (hatched), and treated-and-recovered (closed bars) hepatocyte membranes. **Panel B:** Hepatocyte membranes assayed in panel A were solubilized with 1% cholate and spun at 100,000xg. The indicated amounts of diluted extracted membrane proteins were added to adenylyl cyclase assays containing 10  $\mu$ g of membranes from S49 *cyc* cells, which lack  $G_s$ . NaF-stimulated adenylyl cyclase activity, due to hepatocyte  $G_s$  stimulation of the S49 cell adenylyl cyclase, is shown versus the amount of hepatocyte extract protein added. **Panel C:** The ratio of hepatocyte NaF/ $G_s$  activity in membrane cholate suspension and the resulting 100,000xg cleared extract is shown for each cell treatment. Assays were performed in parallel with those in Panel B.

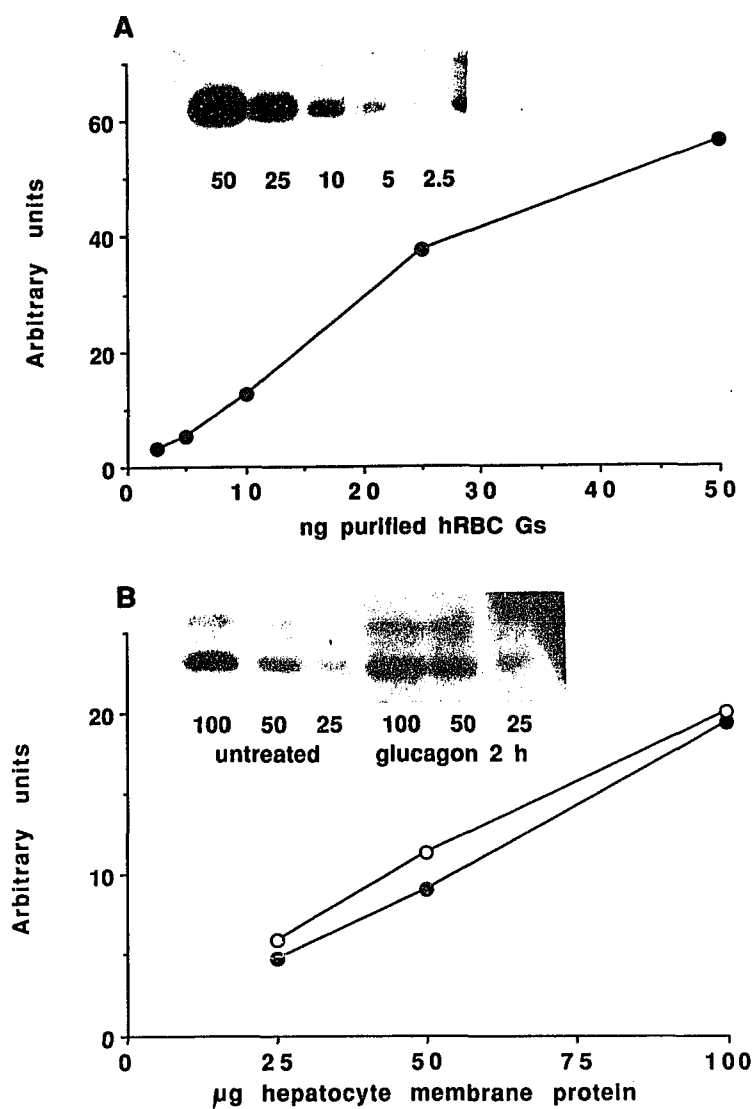


### Immunoblotting for G<sub>s</sub>- $\alpha$

To determine directly whether this decrease in G<sub>s</sub> activity in glucagon-treated cell membranes is due to loss of G<sub>s</sub>  $\alpha$ -subunit protein as compared with untreated cell membranes, quantitative immunoblotting using antipeptide antisera to measure  $\alpha_s$  protein in untreated and glucagon-treated hepatocyte membranes was performed. Using G<sub>s</sub> purified from human erythrocytes, a standard curve for recognition of  $\alpha_s$  in immunoblots by antiserum A-584 was prepared (Figure 4-4, panel A). Various concentrations of untreated and glucagon treated hepatocyte membrane proteins were separated by SDS-PAGE, transferred to nitrocellulose sheets and probed for A-584 immunoreactivity in the linear range of the assay. Quantitation of the 42 kDa immunoreactive band autoradiographic intensity is shown in panel B; the 80-90% lower abundance of the larger (52 kDa) form of  $\alpha_s$  made its quantitation less precise, so it was not included in the densitometric analysis. Glucagon-treated cell membranes were observed to contain essentially the same level of  $\alpha_s$  as did untreated cell membranes in three independent experiments, and in the experiment shown here slightly more rather than less. Hepatocyte membranes appear to contain the equivalent immunoreactivity of 10 ng of  $\alpha_s$  per 100  $\mu$ g of membrane proteins.

Given the linearity of the standards and the clear distinction between their two-fold differences, a 25% difference in membrane G<sub>s</sub>- $\alpha$  protein levels should be detectable. The relatively low level of G<sub>s</sub> protein and activity in chick hepatocyte membranes (eg., Padrell *et al*, 1987), the unknown degree of crossreactivity of the anti-bovine  $\alpha_s$  peptide antiserum A-584 with chicken  $\alpha_s$ , and the relatively small predicted difference (20-25%) in G<sub>s</sub> levels may be responsible for the inability to discern alterations in G<sub>s</sub> levels using these

Figure 4-4. Quantitative immunoblotting for  $G_s\alpha$  protein. Hepatocytes were incubated in the presence of 1  $\mu$ M glucagon for 2 hours prior to homogenization. 25, 50 and 100  $\mu$ g of total membrane protein from untreated and glucagon-treated cells, as well as known amounts of purified human erythrocyte  $G_s$ , were subjected to SDS-PAGE and transferred to a nitrocellulose sheet. The sheet was incubated with 1:500 dilution of antiserum A-584, followed by [ $^{125}$ I]goat anti-rabbit IgG. Bound antibodies were visualized by autoradiography. *Panel A*: Standard curve for recognition of known quantities of purified human  $G_s\alpha$  by A-584, derived from densitometry of the autoradiogram shown in *inset*. *Panel B*: Recognition of hepatocyte membrane  $G_s$  by A-584. Densitometry of the lower 42 kDa  $G_s\alpha$  bands in the *inset* is plotted versus the amount of hepatocyte membrane protein used. Closed circles are from untreated cell membranes, and open circles from glucagon-treated hepatocyte membranes.



conditions. Although the antiserum appears sensitive enough to detect such small differences at this level, the gel needed to be loaded nearly to capacity with membrane proteins (100  $\mu$ g) in order to see a clear signal and the background was rather high due to the large amount of protein.

#### Analysis of pertussis toxin substrates

$G_i$  and  $G_o$  proteins are substrates for ADP-ribosylation by pertussis toxin; this covalent modification prevents coupling of receptors to these G proteins without affecting their regulation by guanine nucleotides (Van Dop *et al*, 1984b; Murayama & Ui, 1984). Using [ $^{32}$ P]NAD $^{+}$  as a substrate, pertussis toxin can specifically label the  $G_i$  and  $G_o$  proteins in cell membranes. Labeling of chick hepatocyte membranes with [ $^{32}$ P]NAD $^{+}$  and pertussis toxin reveals the presence of two closely-spaced 40-41 kDa substrate ( $G_i$ ) proteins, which appear as a single band at this exposure (Figure 4-5). In this experiment, equal amounts of three different batches of untreated and glucagon-treated hepatocyte membranes were labeled. In none of these membrane preparations was there any significant difference in the apparent levels of the pertussis toxin substrates (measured as a single band). Glucagon-induced desensitization does not appear to alter the amount of pertussis toxin-substrate  $G_i$  proteins in hepatocyte membranes.

#### Immunoblotting for $G_i$ - $\alpha$

To determine more directly whether there was any change in  $\alpha_i$  levels following glucagon treatment,  $\alpha_i$  immunoreactivity was determined in immunoblots using antiserum AS/6, which recognizes all three  $\alpha_i$  subtypes (Falloon *et al*, 1986). The experiment in

Figure 4-5. Pertussis toxin catalyzed ADP-ribosylation of hepatocyte membrane proteins. Hepatocytes were incubated for 2 h in the absence and presence of 1  $\mu$ M glucagon prior to homogenization. Lane 1 contains 5  $\mu$ g of untreated cell membranes incubated with [ $^{32}$ P]NAD $^{+}$  in the absence of pertussis toxin. Membranes (5  $\mu$ g) from three independent preparations of untreated (lanes 2,4,6) and glucagon-treated (3,5,7) cells were incubated in the presence of activated pertussis toxin and [ $^{32}$ P]NAD $^{+}$  for 30 min. Reactions were precipitated with TCA and the pellets resuspended and separated by SDS-PAGE prior to exposure to X-ray film overnight.

**Chick Hepatocyte Membranes**  
**Pertussis Toxin**  
**ADP-Ribosylation**

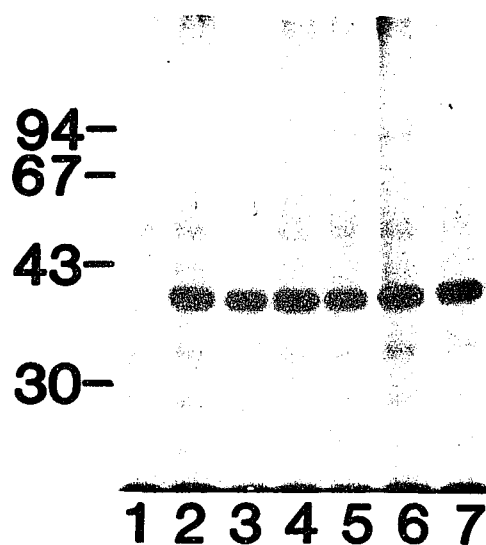


Figure 4-6. Quantitative immunoblotting for  $G_i\alpha$  proteins. Hepatocytes were incubated in the presence of  $1\ \mu\text{M}$  glucagon for 2 hours prior to homogenization.  $100\ \mu\text{g}$  of total membrane protein from untreated and glucagon-treated cells, as well as known amounts of purified human erythrocyte  $G_i$ , were subjected to SDS-PAGE and transferred to a nitrocellulose sheet. The sheet was incubated with 1:100 dilution of antiserum AS/6, followed by [ $^{125}\text{I}$ ]goat anti-rabbit IgG. Bound antibodies were visualized by autoradiography. *Panel A*: Standard curve for recognition of known quantities (ng) of purified human  $G_i\alpha$  (mainly  $G_{i3}$ ) by AS/6, derived from densitometry of the autoradiogram shown in *inset*. *Panel B*: Recognition of hepatocyte membrane  $G_i$  proteins by AS/6. Densitometry of the 40-41 kDa  $G_i\alpha$  bands in the *inset* is shown for untreated (C) and glucagon-treated (D) hepatocyte membranes.

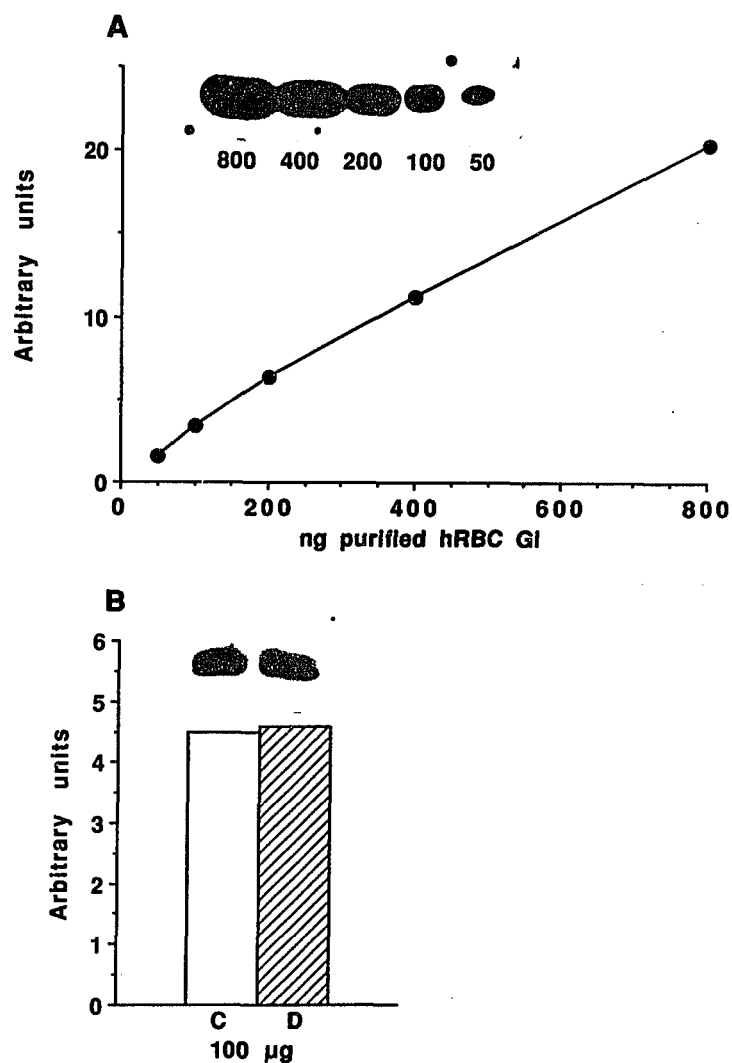


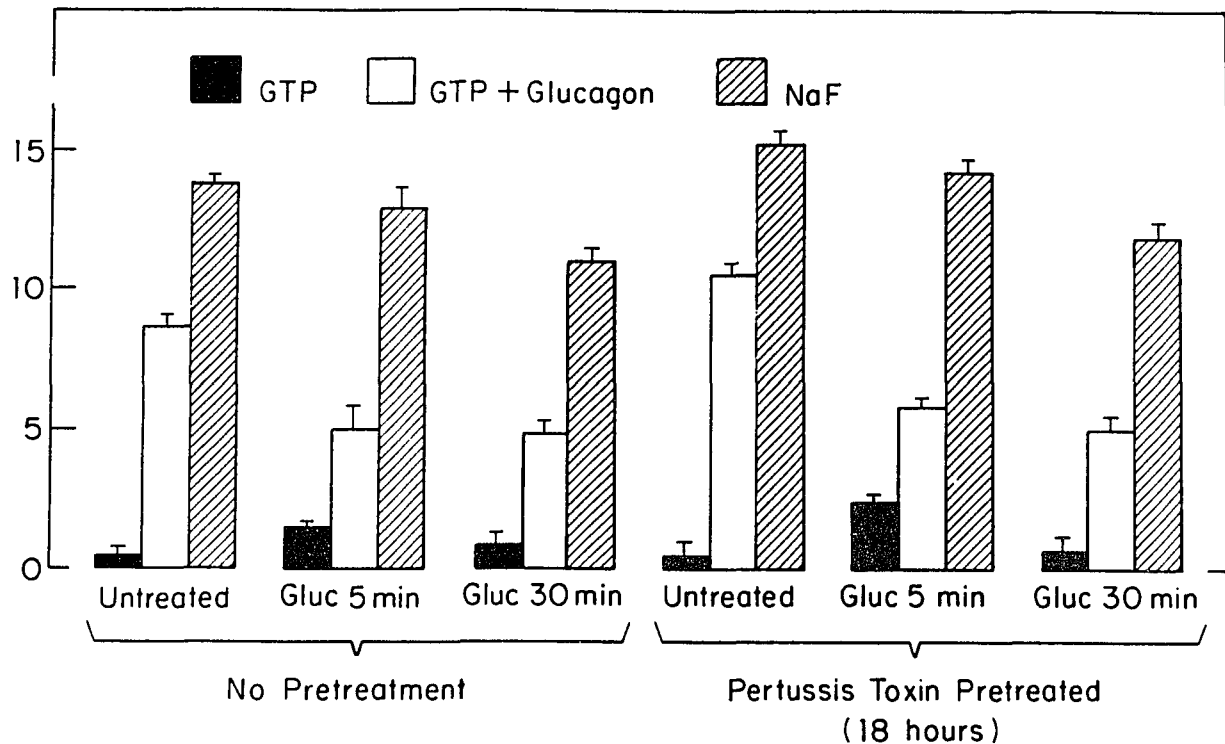
Figure 4-6, panel A, shows that AS/6 can be used to quantitatively detect  $\alpha_i$  subunits (mainly  $\alpha_{i3}$ ) purified from human erythrocytes. Under linear assay conditions, there were no observable differences in the levels of the two 40-41 kDa  $\alpha_i$ -subunits (treated as a single band, since they are too close to resolve by densitometry) as measured using antiserum AS/6 in untreated and glucagon-treated hepatocyte membranes (panel B). Hepatocyte membranes contain the equivalent of 50-60 ng of  $\alpha_i$ -immunoreactivity per 100  $\mu$ g of membrane protein.

#### Functional effects of pertussis toxin treatment

Pertussis toxin pretreatment of rat hepatocytes had been reported to block the development of glucagon-induced (homologous) desensitization (Heyworth *et al*, 1984a). To assess the functional involvement of these pertussis toxin substrate ( $G_i$ ) proteins in the glucagon-induced desensitization, hepatocytes were incubated for 18 hour with 200  $\mu$ g/ml unactivated pertussis toxin prior to treatment with glucagon. Adenylyl cyclase activities for one set of membranes from pertussis toxin-treated hepatocytes are shown in Figure 4-7. Prior treatment with toxin had no effect on the appearance of either the homologous (as measured by glucagon stimulation) or the heterologous (as measured by NaF stimulation) desensitizations, although membranes from pertussis toxin-treated cells do show a slight increase in all stimulated adenylyl cyclase activities. As a control to ensure that pertussis toxin had entered the cells, membranes were subjected to labeling by pertussis toxin-catalyzed ADP-ribosylation using [ $^{32}$ P]NAD $^{+}$ . Membranes from pertussis toxin treated cells showed an complete absence of labeling of the  $G_i$  bands compared to those from untreated cells from the same toxin pretreatment experiment (data not shown).

Figure 4-7. Effect of pertussis toxin pretreatment on development of glucagon-induced desensitization. Hepatocytes were incubated for 18 h with 1  $\mu$ g/ml pertussis toxin (inactive holotoxin). Cells were treated with 1  $\mu$ M glucagon for an additional 5 or 30 min prior to homogenization. Membranes were assayed for adenylyl cyclase activity (pmol/min/mg membrane protein) stimulated by GTP (closed bars), GTP and glucagon (open bars), and NaF (hatched bars).

### Chick Hepatocytes



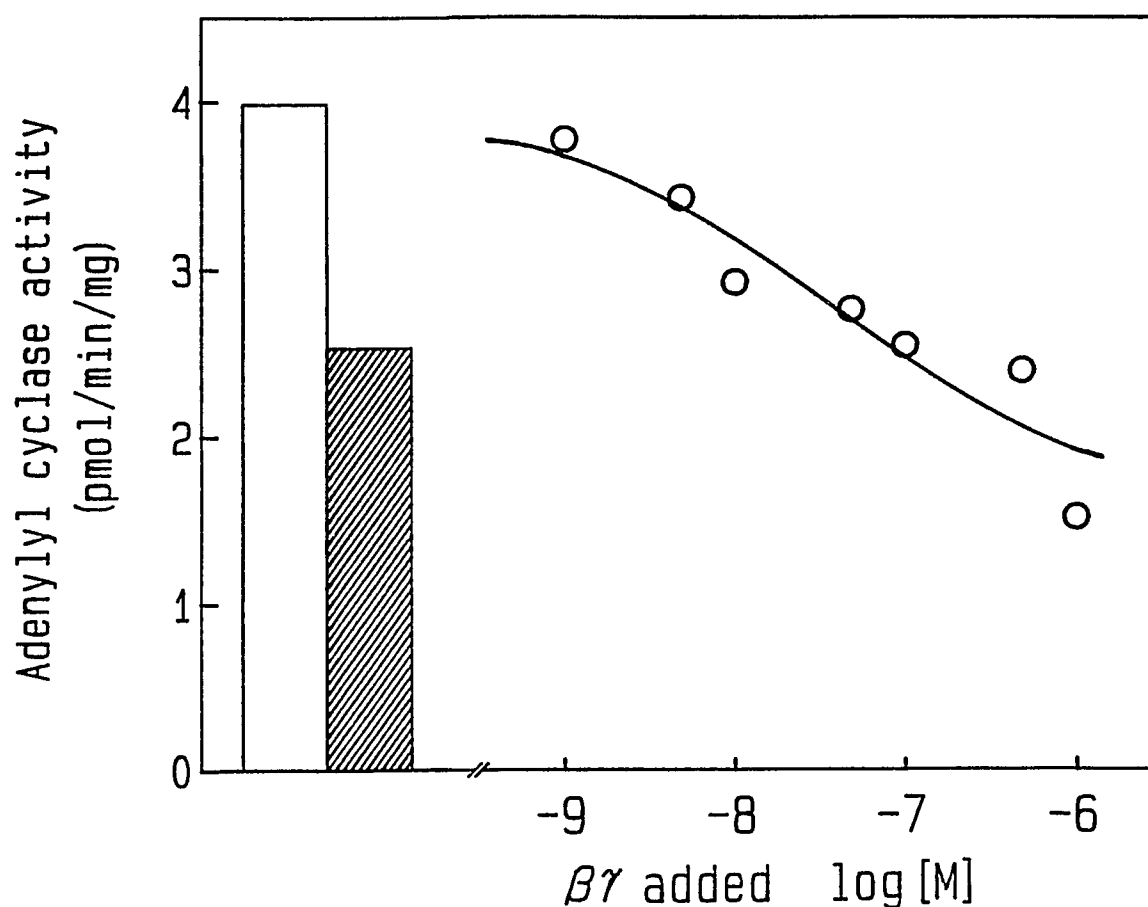
### Effects of $\beta\gamma$ -subunits on adenylyl cyclase activity

Since the levels of  $\alpha_i$  proteins appear to be unchanged in heterologous desensitization, changes in the level of  $\beta\gamma$ -subunits are another alternative explanation for decreased activity of  $G_s$ -regulation of adenylyl cyclase in heterologous desensitization. Increased levels of the  $\beta\gamma$ -subunits of G proteins attenuate  $G_s$  stimulation of adenylyl cyclase, presumably by forcing a reassociation of the active  $\alpha_s$  subunit with the excess  $\beta\gamma$  subunits (Northup *et al*, 1983b). (However, cloned type 2 and 4 adenylyl cyclases appear to be *stimulated* directly by  $\beta\gamma$  subunits in the presence of activated  $G_s$ , see Chapter 7). In order to estimate the magnitude of change in  $\beta\gamma$ -subunit levels which could reduce NaF-stimulated adenylyl cyclase activity in hepatocyte membranes by the extent seen following glucagon treatment, varying concentrations of  $\beta\gamma$ -subunits purified from human erythrocytes were mixed with untreated hepatocyte membranes prior to assay for NaF stimulated adenylyl cyclase activity. As can be seen in Figure 4-8, a 100-fold increase in added  $\beta\gamma$ -subunits (from 1 nM to 100 nM) is required to reduce NaF-stimulated adenylyl cyclase activity by 25%, comparable to the glucagon-induced reduction in activity in desensitized cell membranes. (Further, the obvious inhibition of hepatocyte NaF-stimulated adenylyl cyclase activity by added  $\beta\gamma$  subunits, rather than stimulation, indicates that little type 2 or 4 adenylyl cyclase is likely to be present in these cells).

### Immunoblotting for $\beta_1$ Subunits of G Proteins

Quantitative immunoblotting using the G protein  $\beta_1$ -subunit ( $\beta_{36}$ -subunit) specific antiserum U-49 was used to compare directly the relative levels of the  $\beta_1$  protein in

Figure 4-8. Effect of exogenous  $\beta\gamma$  subunits of G proteins on NaF-stimulated adenylyl cyclase activity of hepatocyte membranes.  $\beta\gamma$ -subunits purified from human erythrocytes (gift of J. Codina) were diluted in 25 mM HEPES, 1 mM EDTA, 300 mM NaCl, 0.1% Lubrol, and 30% ethylene glycol (pH 8.0). Diluted  $\beta\gamma$  subunits (or buffer only) were added to untreated hepatocyte membranes (10  $\mu\text{g}$ ) in a standard adenylyl cyclase reaction containing 10 mM NaF but lacking  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ . This pre-assay mixture was incubated for 10 min at 32°C to allow protein association with the membranes. At this time, the  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  was added, and the assay incubation continued for 10 min prior to purification and quantification of cAMP. Data is shown as NaF-stimulated adenylyl cyclase activity versus concentration of purified  $\beta\gamma$  subunits added. NaF-stimulated adenylyl cyclase activity of untreated hepatocyte membranes assayed in the presence of buffer only is shown as the open bar. In addition, membranes from hepatocytes treated for 2 h with 1  $\mu\text{M}$  glucagon were also assayed in the same manner, with addition of buffer only (hatched bar).



untreated and glucagon-treated hepatocyte membranes. The linearity of the immunoblotting assay is shown in the upper panel of Figure 4-9 using holo- $G_i$  purified from human erythrocytes. Autoradiographic intensity is linear up to 1  $\mu\text{g}$  of  $G_i$  protein (400 ng of  $\beta$ -subunit protein). Under linear assay conditions, we are unable to see any difference between the levels of  $\beta_1$ -subunits in untreated and glucagon-treated cell membranes (lower panel). Using this standard curve, hepatocyte membrane contain the equivalent of 25 ng of  $\beta_{36}$  protein immunoreactivity per 100  $\mu\text{g}$  of membrane protein. By comparison with Figure 4-8, it is possible to estimate that 10  $\mu\text{g}$  of membranes in the standard 50  $\mu\text{l}$  adenylyl cyclase assay gives just under 1 nM  $\beta$ -subunits, and that this must be increased to 100 nM by addition of exogenous  $\beta\gamma$ -subunits in order to achieve a 25% decrease in NaF-stimulated adenylyl cyclase activity. Such major alterations in  $\beta_{36}$ -protein levels are clearly not observed following glucagon treatment of hepatocytes. These observations indicate that heterologous desensitization is not associated with large changes in the levels of  $\beta\gamma$ -subunits, and the magnitude of change required for such a mechanism to be operative is very great.

#### Supplementation of membranes with $G_s$

Since I was unable measure changes in the levels of G protein subunits which might account for the heterologous desensitization and since decrease in  $G_s$  activity is the only measurable change associated with heterologous desensitization, I tested whether addition of exogenous  $G_s$  could restore NaF stimulation of adenylyl cyclase in glucagon-treated cell membranes and thus "reverse" heterologous desensitization, as had been observed by Rich *et al* (1984) in glucagon-treated MDCK cells. Under conditions where

Figure 4-9. Quantitative immunoblotting for the  $\beta_{36}$  subunit of G proteins. Hepatocytes were incubated in the presence of 1  $\mu$ M glucagon for 2 hours prior to homogenization. Ten and 100  $\mu$ g of total membrane protein from untreated and glucagon-treated hepatocytes, as well as known amounts of purified human erythrocyte holo- $G_i$ , were subjected to SDS-PAGE and transferred to a nitrocellulose sheet. The sheet was incubated with 1:10,000 dilution of antiserum U-49, followed by [ $^{125}$ I]goat anti-rabbit IgG. Bound antibodies were visualized by autoradiography. *Panel A*: Standard curve for recognition of known quantities (ng) of purified human  $G_i$ s by U-46, derived from densitometry of the autoradiogram shown in *inset*. *Panel B*: Recognition of hepatocyte membrane  $\beta_{36}$  protein by antiserum U-46. Densitometry of the 36 kDa  $\beta_{36}$  bands in the *inset* is shown, for the indicated amounts of untreated (C) and glucagon-treated (D) hepatocyte membrane proteins.

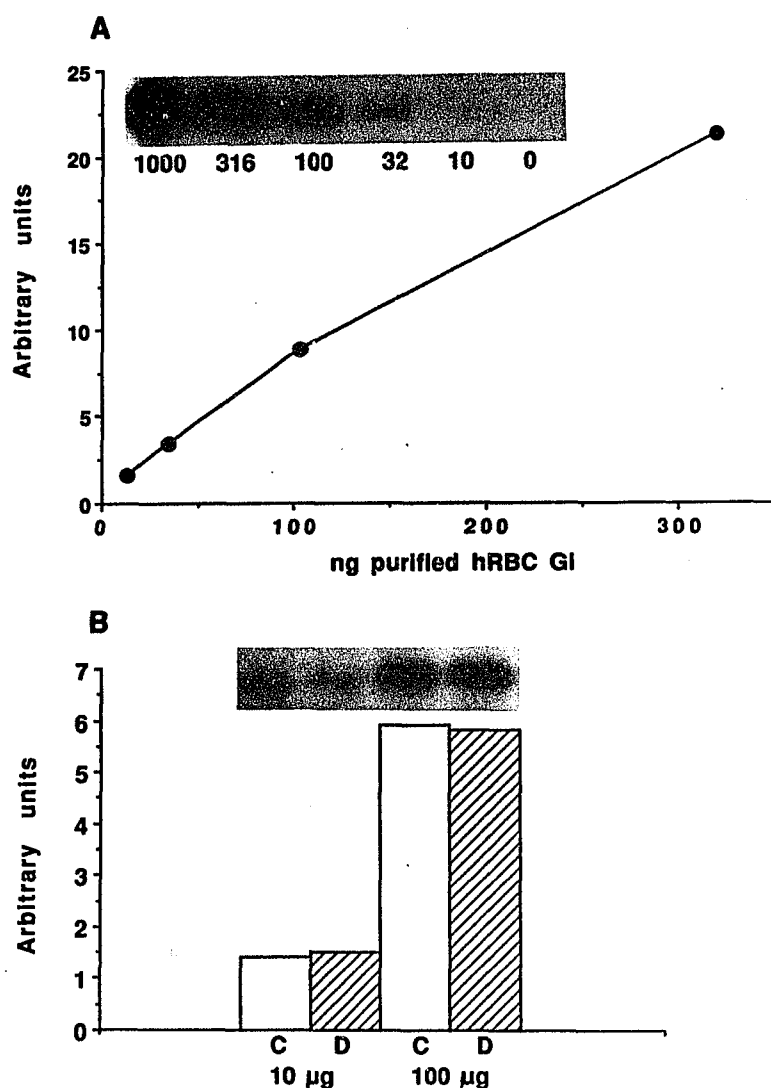
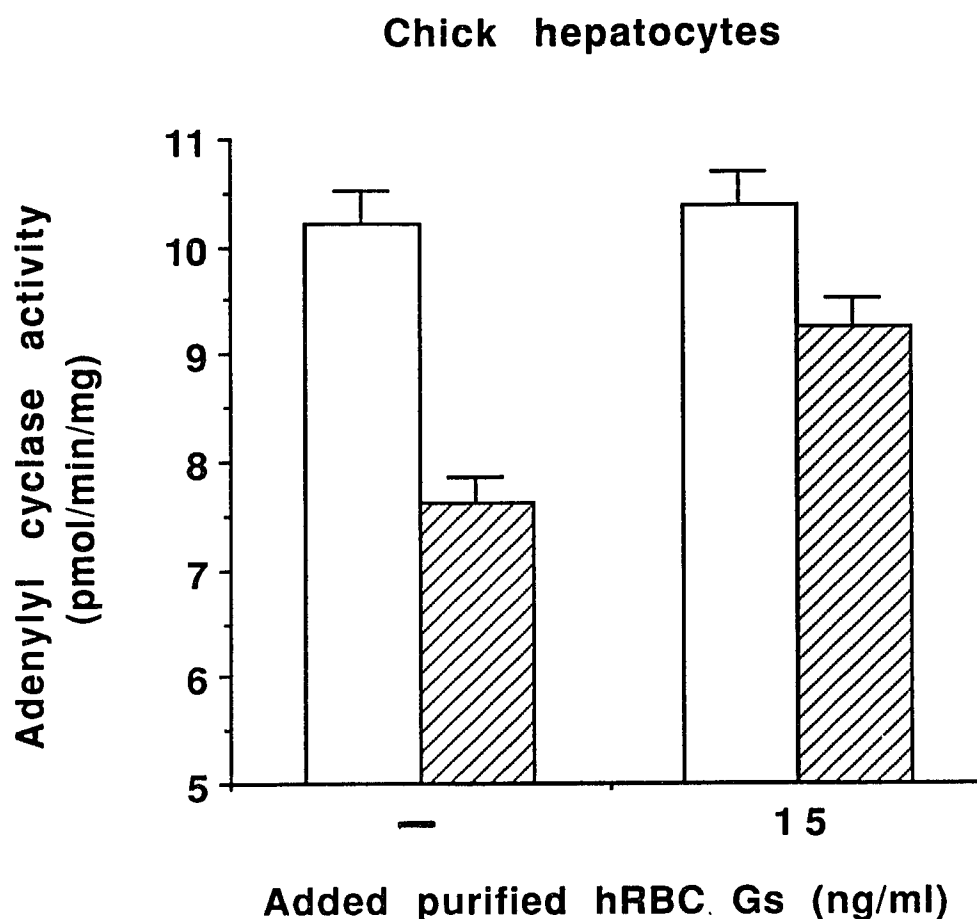


Figure 4-10. Effect of exogenous  $G_s$  on NaF-stimulated adenylyl cyclase activity of glucagon-desensitized membranes. Hepatocytes were incubated in the presence of  $1\ \mu\text{M}$  glucagon for 2 hours prior to homogenization.  $G_s$  purified from human erythrocytes (gift of J. Codina) was diluted in 25 mM Tris, 1 mM EDTA, 1 mM DTT, and 0.05% BSA (pH 8.0). Hepatocyte membranes ( $13\ \mu\text{g/ml}$ ) were mixed with an equal volume of diluted  $G_s$  or buffer and incubated for 20 min on ice. This reconstituted  $G_s$ -membrane mixture ( $15\ \mu\text{l}$ ) was added to a standard adenylyl cyclase assay and incubated for 10 min at  $32^\circ\text{C}$ . Adenylyl cyclase activity stimulated by 10 mM NaF is shown for untreated (open bars) and glucagon-treated (hatched bars) hepatocyte membranes, in the absence and presence of 15 ng/ml (200 pM) added  $G_s$  protein.



addition of  $G_s$  (0.6 nM) does not increase NaF-stimulated adenylyl cyclase activity of untreated membranes,  $G_s$  addition to glucagon-treated membranes increased stimulation by NaF to levels near those seen in untreated cell membranes (Figure 4-10). Higher concentrations of  $G_s$  were found to increase basal and stimulated adenylyl cyclase activities in both untreated and glucagon-treated hepatocyte membranes. Since low levels of exogenous  $G_s$  appear to at least partially reverse the desensitization observed in hepatocyte membranes following cell treatment with glucagon, alterations of the  $G_s$  protein may mediate the heterologous desensitization measured through NaF-stimulation of adenylyl cyclase.

### Summary

Treatment of chick hepatocytes with glucagon induces a partial (20-25%) loss of NaF-stimulated adenylyl cyclase activity. This heterologous desensitization response must be due to post-receptor alteration(s) of the adenylyl cyclase system, since only  $G_s$  and the adenylyl cyclase enzyme itself are required to express NaF stimulated adenylyl cyclase activity (Northup *et al*, 1983a). Heterologous desensitization of adenylyl cyclase can also be observed following hepatocyte treatment with other hormones which activate adenylyl cyclase through  $G_s$  in these cells, such as vasoactive intestinal peptide (VIP), isoproterenol and prostaglandin  $E_1$ , but not with hormones which do not activate adenylyl cyclase (angiotensin II, vasopressin). The similar reduction of NaF-stimulated adenylyl cyclase activity by treatment with glucagon and these other stimulatory hormones indicates that a common mechanism may be responsible for the heterologous desensitization caused by these hormones. However, this effect is only partially mimicked by treatment of

hepatocytes with a stable and membrane-permeable cAMP analog, 8-Br-cAMP, indicating that the desensitization is not entirely cAMP-dependent (or cAMP-independent).

This heterologous desensitization occurs very rapidly following addition of hormone, requiring only 15-30 min to reach a maximal loss of adenylyl cyclase activity. Removal of glucagon from treated cells results in rapid (30 min) restoration of NaF-stimulated adenylyl cyclase activity. Following rapid treatment (2 min) and removal of glucagon from the hepatocytes, membranes exhibit a loss of NaF stimulation of adenylyl cyclase within 5 min, which recovers completely in the same time-frame. The rapidity and reversibility of this heterologous desensitization limit the types of modulations which may be responsible to those which are rapid enough to account for these observed time-courses. From studies of the effect of cycloheximide, no new protein synthesis appears to be required for the occurrence of or recovery from heterologous desensitization.

Direct measurement of the  $G_s$  activity resident in plasma membrane fractions prepared from untreated and glucagon-treated hepatocytes by reconstitution of membrane detergent extracts with adenylyl cyclase in membranes from the  $G_s$ -deficient *cyc*<sup>-</sup> S49 lymphoma cell line indicates that reduced NaF-stimulated adenylyl cyclase activity of membranes following glucagon treatment is associated with a loss of  $G_s$  activity in the membranes. Quantitative immunoblotting to directly determine whether such lost activity is due to proportional loss of  $\alpha$ -subunits of the  $G_s$  protein was inconclusive, due to the low abundance of the  $G_s$  protein and (perhaps) the ability of the chicken  $G_s$  to be recognized by the bovine  $G_s$  peptide antiserum. Alterations in the levels of  $G_i\alpha$  or the  $\beta$ -subunits of G proteins were not observed, and uncoupling of  $G_i$  proteins from receptors by treatment with pertussis toxin had no apparent effect on the development of either

homologous or heterologous desensitization following glucagon treatment. However, readdition of small amounts of purified  $G_s$  to membranes from glucagon-treated hepatocytes leads to a partial recovery of both hormone and NaF-stimulated adenylyl cyclase activity. Since there is a loss of  $G_s$  activity following glucagon treatment which can be overcome by addition of  $G_s$  to the membranes, it appears that  $G_s$  is a major locus of the heterologous alteration in this system.

Glucagon-induced heterologous desensitization has been reported in cultured rat hepatocytes (Gurr & Ruh, 1980; Noda *et al*, 1984; Refsnes *et al*, 1989), cultured rat Sertoli cells (Attramadal *et al*, 1988) and the canine kidney MDCK cell line (Rich *et al*, 1984). None of the studies used NaF or other post-receptor stimulators to define the time-course of the heterologous component alone, but measured mainly hormone-stimulated activity (which measures both homologous and heterologous components). In most instances, full heterologous desensitization appeared to require 1-2 h of glucagon treatment; the desensitization reported by Noda *et al* (1984) appeared to require >9 h to reach its maximal extent. Gurr & Ruh (1980) also characterized recovery following glucagon withdrawal, and found that 1 h was sufficient to relieve the desensitized state.

The glucagon-induced heterologous desensitization reported by Noda *et al* (1984) in rat hepatocytes appears most similar to that seen in chick hepatocytes, although several methodological differences obscure more than enlighten. These workers found no difference in cAMP accumulation in control and glucagon-treated hepatocytes which had been treated with cholera toxin to persistently activate the  $G_s$  protein. Further, labeling of the  $G_s$  in membranes from control and glucagon-treated cells using cholera toxin and [ $^{32}$ P]NAD revealed no change in the apparent level of the  $G_s$  protein. Neither assay is

quantitative or very sensitive to small changes in  $G_s$ . Glucagon treatment (but not a cAMP analog) reduced both NaF and forskolin stimulated adenylyl cyclase activities, but since the  $G_s$  activity of the plasma membranes was not directly determined, it is not possible to assign the locus of this alteration to  $G_s$  or adenylyl cyclase. This pattern of loss is consistent with the loss of a fraction of the membrane  $G_s$  activity, however, as seen in chick hepatocytes.

The mechanism for such an agonist-induced loss of  $G_s$  activity in the cell membrane is currently unknown. The rapid time-course of the loss of activity and its recovery demand a readily reversible change, which clearly excludes new protein synthesis. Many post-translational modifications of proteins (myristoylation, palmitoylation, isoprenylation, sulfation, glycosylation) are not sufficiently reversible (or known to be hormone regulated) to account for this loss of activity. Endogenous protein ADP-ribosylation and de-ADP-ribosylation of proteins has been reported (Moss *et al*, 1988; Tanuma *et al*, 1988; Peterson *et al*, 1990), but ADP-ribosylation of  $G_s\alpha$  by cholera toxin activates rather than inhibits its activity. Phosphorylation is frequently proposed as a mechanism. Protein kinase A or perhaps another of the kinases it activates in liver could phosphorylate  $G_s$  and reduce its activity. However, the sequence of  $G_s\alpha$  contains no predicted protein kinase A phosphorylation sites (Bray *et al*, 1986), and attempts to demonstrate phosphate incorporation into  $G_s$  subunits upon desensitization have yielded negative results (K. Rich and RI, unpublished).

A distinct possibility is that the levels of the  $G_s$  protein is regulated by the cell, and hormone treatment leads to reduced  $G_s$  protein in the plasma membrane. Direct measurement of  $G_s$  protein levels by immunoblotting was unable to demonstrate a

significant loss of  $G_s$  protein. It is not clear that "intracellular" pools of uncoupled  $G_s$  would necessarily be removed from the plasma membrane, but could be in domains segregated from receptors and adenylyl cyclase, or in plasma membrane associated vesicles. In such cases, no loss of  $G_s$  protein could be detected by immunoblotting techniques. Further analysis of various membrane and vesicle fractions of glucagon-treated hepatocytes suggested that some  $G_s$  activity could be found within "endosome" fractions of the cell, but the low levels of activity and low yield of vesicle fractions precluded definitive analysis to ensure that this is not due to small amounts of plasma membrane contamination of vesicle fractions.  $G_s$  has been reported to be transferred from the plasma membrane to intracellular membrane fractions in response to agonists (Haraguchi & Rodbell, 1990).  $G_i$  proteins have also been associated with agonist-induced translocation from the plasma membrane (Retrosen *et al*, 1988; Crouch *et al*, 1989; Milligan & Green, 1991; Reithmann *et al*, 1991). The mechanistic basis for such translocation is unknown, but such a mechanism would explain lost  $G_s$  activity following glucagon treatment of chick hepatocytes.

Glucagon-induced heterologous desensitization described in the MDCK cell line by Rich *et al* (1984) appears quite different from any other so far described in any system. Glucagon induces a substantial loss (30-40%) of hormone- and NaF-stimulated adenylyl cyclase activity. Treatment with 8-Br-cAMP also reduced hormonal stimulation by 30%, but had marginal effects on NaF stimulation. No change in  $G_s$  activity or cholera toxin labeling of  $G_s$  proteins was observed in membranes from glucagon-treated cells. Addition of 7.5 nM  $G_s$  to glucagon-treated membranes restored control levels of NaF-stimulation of adenylyl cyclase, but had no effect on lost hormonal stimulation. In

this system, the major mode of heterologous desensitization appears to result from the cAMP-dependent phosphorylation and inactivation of the receptors. A cAMP-independent effect on  $G_s$ -dependent activity does not result from direct loss of membrane  $G_s$  activity, but was shown to be associated with increased labeling of  $G_i$  proteins by pertussis toxin. The mechanistic basis for this observation is unknown.

The partial desensitizing effect of cAMP analogs compared to glucagon observed in the chick hepatocyte has also been noted in rat hepatocytes (Gurr & Ruh, 1980; Refsnes *et al*, 1989), and a very small effect was seen in MDCK cells (Rich *et al*, 1984). Thus, the heterologous desensitization appears to be composed of two distinct alterations. In the hepatocyte, studies described in the next chapter of the effects of reconstitution of exogenous  $G_s$  with desensitized membranes allowed the determination that this cAMP-independent modulation appears to be due to regulation of the activity of  $G_s$  in the plasma membrane (other studies have not explicitly examined this). The locus of the cAMP-dependent step was not known, and I set out to define it.

## CHAPTER 5

### HETEROLOGOUS DESENSITIZATION: FUNCTIONAL REGULATION OF ADENYLYL CYCLASE

Direct regulation of adenylyl cyclase activity by factors other than  $G_s$  has been clearly demonstrated for very few proteins or compounds. The direct interaction of forskolin, calmodulin and adenosine-like P-site agonists has been clearly established by both affinity purification and by characterization of the properties of the cloned and expressed type 1 enzyme (Seamon & Daly, 1981a; Pfeuffer & Metzger, 1982; Westcott *et al*, 1979; Johnson, 1982; Tang *et al*, 1990). Even direct regulation by  $G_i$  proteins has yet to be unequivocally established for a purified or cloned adenylyl cyclase, irrespective of arguments about whether the  $\alpha$  or  $\beta\gamma$ -subunits mediate this inhibition.

The potential role of phosphorylation of the adenylyl cyclase enzyme in its regulation has been very seldom addressed. Other cellular signaling molecules, such as phospholipase C forms and  $Ca^{2+}$  channels, are known to be targets for phosphorylation by second messenger-regulated protein kinases A and C (Kim *et al*, 1989; Schmid *et al*, 1985). Evidence for the direct stimulatory modulation adenylyl cyclase by protein kinase C has been obtained in several systems (Bell *et al*, 1985; Yoshimasa *et al*, 1987). However, little substantial evidence of a role for cAMP-dependent regulation of adenylyl cyclase has been reported. During studies of glucagon-induced desensitization of the adenylyl cyclase system in chick hepatocytes, I have uncovered evidence for a cAMP-

dependent reduction in the ability of adenylyl cyclase to be stimulated by  $G_s$ . The evidence obtained thus far is circumstantial, but is the first suggestion of the physiological modulation of adenylyl cyclase itself by protein kinase A-mediated phosphorylation.

#### $G_s$ supplementation of desensitized membranes

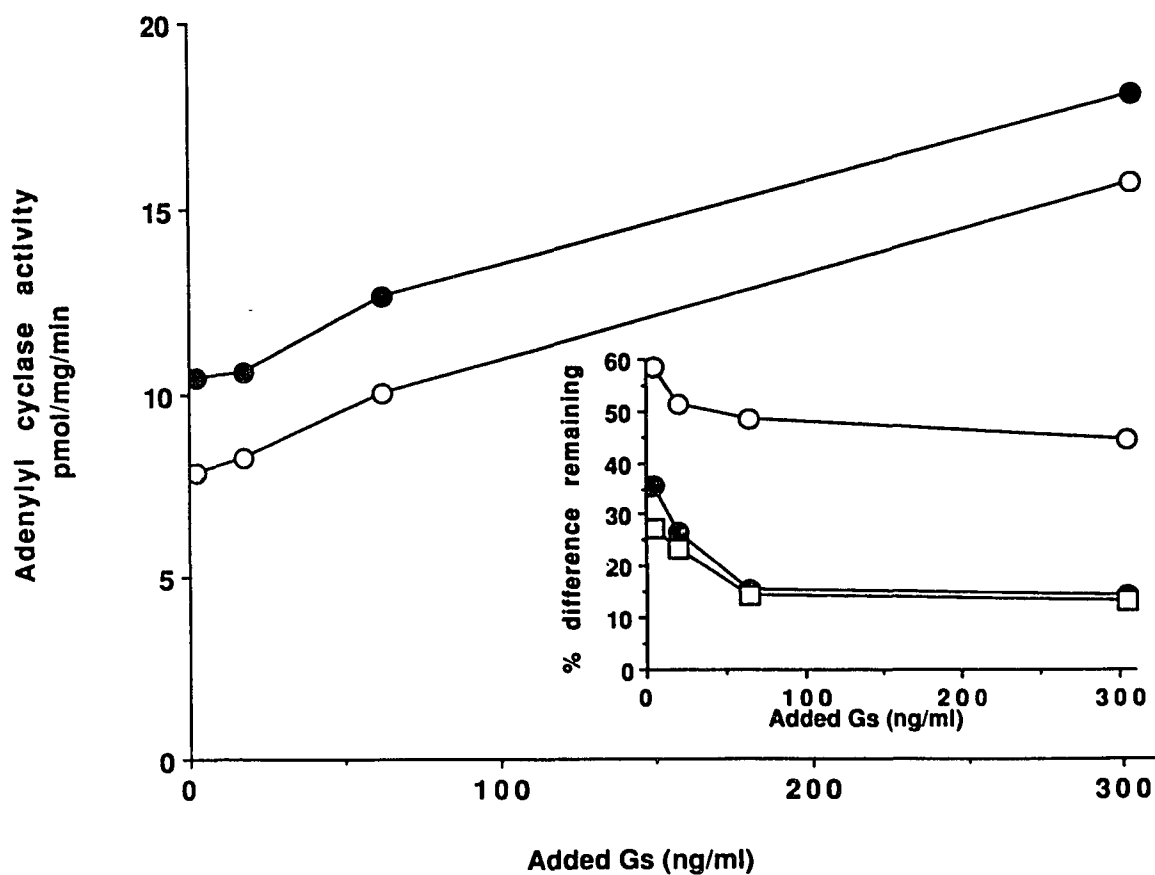
The  $G_s$  activity of glucagon-treated chick hepatocyte membranes is reduced compared to  $G_s$  activity from untreated cell membranes, as measured by reconstitution of hepatocyte membrane detergent extracts with *cyc*<sup>-</sup> S49 cell membrane adenylyl cyclase (Figure 4-3). If the heterologous desensitization were due *solely* to loss of  $G_s$  activity in the plasma membrane, the desensitization should be reversed by adding purified  $G_s$  to the glucagon-treated hepatocyte membranes. When quite low concentrations (200 pM) of purified  $G_s$  was preincubated with desensitized hepatocyte membranes, some recovery of NaF-stimulated adenylyl cyclase activity in the desensitized cell membranes was observed, without apparent change in the activity of control membranes (Figure 4-10). In the course of these experiments, however, I noted that this recovery was always partial. Even as higher amounts of purified  $G_s$  were added, which imparted increased adenylyl cyclase activity to both control and desensitized cell membranes, a difference in NaF-stimulated adenylyl cyclase activity between the control and glucagon-treated hepatocyte membranes persisted.

This inability of desensitized hepatocyte membranes to respond as robustly as control cell membranes to added  $G_s$  was observed over a range of  $G_s$  concentrations, when adenylyl cyclase was stimulated by NaF, glucagon or a heterologous hormone, VIP

(Figure 5-1). NaF-stimulated adenylyl cyclase activity at various added  $G_s$  concentrations is shown for one set of untreated and glucagon-treated hepatocyte membranes. In the inset, the percentage difference between paired control and glucagon-desensitized membrane adenylyl cyclase activities stimulated by NaF, VIP, or glucagon are plotted versus the final concentration of purified  $G_s$  added. As shown in Figure 4-10, addition of low concentrations of exogenous  $G_s$  partially overcomes the glucagon-induced heterologous desensitization, as measured by stimulation of adenylyl cyclase through the VIP and glucagon receptors or directly by NaF. However, the highest  $G_s$  concentration added in this experiment (4 nM) is insufficient to entirely diminish the heterologous loss of stimulation. Therefore, glucagon treatment produces cell membrane adenylyl cyclase which appears relatively resistant to activation by  $G_s$  compared to untreated cell membranes, whether stimulated directly (NaF) or through a hormone receptor (VIP or glucagon).

The difference between the glucagon stimulation and NaF and VIP stimulation reflect homologous desensitization at the level of the glucagon receptor. The parallel restoration in NaF and VIP stimulated adenylyl cyclase activity indicate that a single alteration probably accounts for the inability of both stimulators to fully activate adenylyl cyclase, even in the presence of "extra"  $G_s$ . Further, the ability of VIP to stimulate adenylyl cyclase as efficiently as NaF at all concentrations of added  $G_s$  indicates that there is no significant multihormonal component of the heterologous desensitization due to protein kinase A phosphorylation of the VIP receptor. A similar experiment in glucagon-treated MDCK cell membranes demonstrated a lack of recovery of glucagon-

Figure 5-1. Dose-dependence of exogenous  $G_s$  on hepatocyte membrane adenylyl cyclase activity. Chick hepatocytes were treated with 1  $\mu$ M glucagon for 2 h prior to homogenization. Membranes were mixed with various concentrations of purified human erythrocyte  $G_s$  or dilution buffer and allowed to sit on ice for 10 min before addition to standard adenylyl cyclase assays. Adenylyl cyclase activity stimulated by NaF is shown versus the final added concentration of  $G_s$  protein for untreated (closed circles) and glucagon-treated cell membranes (open circles). *Inset:* The percentage difference in stimulated adenylyl cyclase activity between untreated and glucagon-treated cell membranes is shown versus the final concentration of  $G_s$ , for NaF- (closed circles), glucagon- (open circles), and VIP- (open squares) stimulated adenylyl cyclase activity.



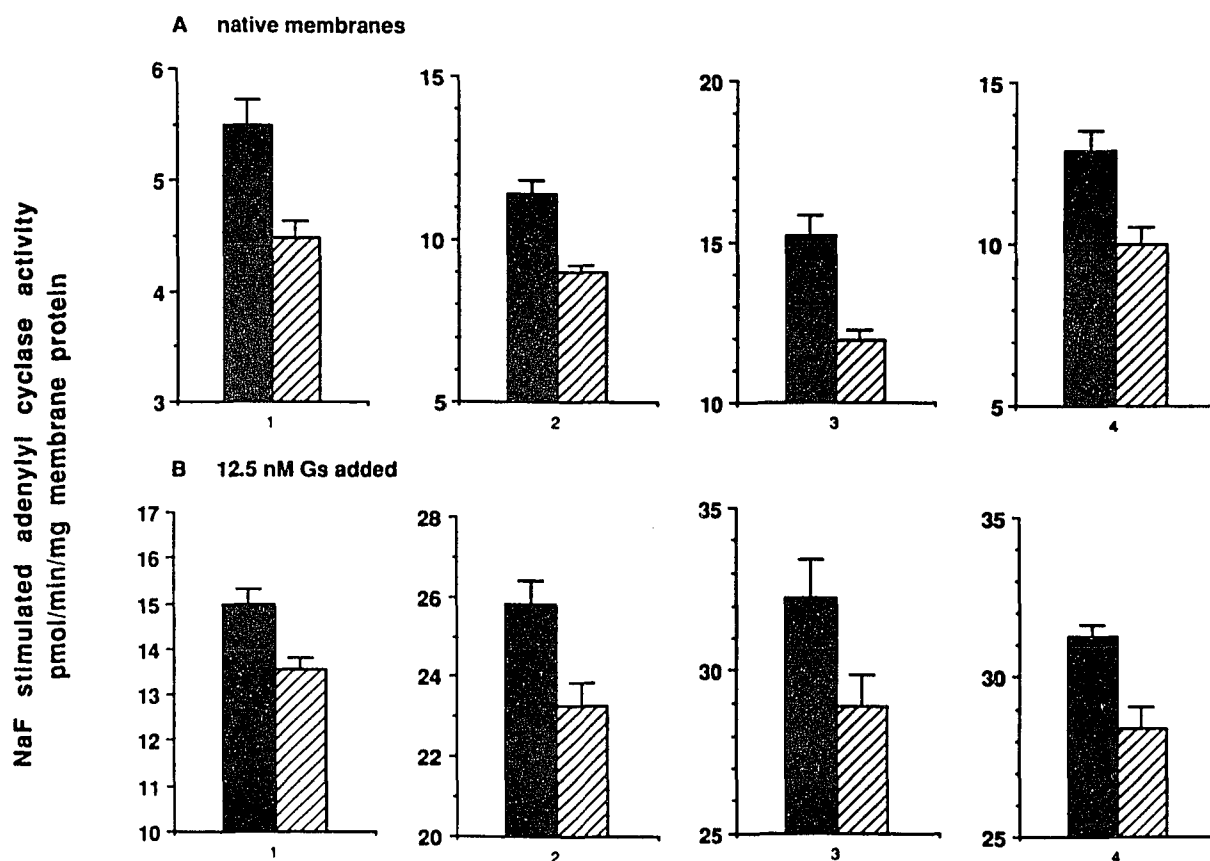
or PGE<sub>1</sub>-stimulated adenylyl cyclase activity upon addition of exogenous G<sub>s</sub> under conditions where NaF-stimulation was completely restored, indicating a block at the level of the hormone receptor coupling to the G<sub>s</sub> most likely due to phosphorylation of the receptor proteins by protein kinase A (Rich *et al*, 1984).

To ensure that this small non-recovery effect was statistically significant, membrane G<sub>s</sub> supplementation experiments were performed many times. In Figure 5-2, four independent preparations of untreated and glucagon-treated hepatocyte membranes were reconstituted with 12.5 nM human erythrocyte G<sub>s</sub> and then assayed for NaF-stimulated adenylyl cyclase activity. In all cases, glucagon-treated cell membranes exhibit small but significant differences in adenylyl cyclase activity compared to untreated cell membranes. Supplementation with exogenous G<sub>s</sub> reveals that glucagon-treated cell membranes, which initially had 75-80% of the activity of untreated cell membranes, can only be restored to 85-90% of untreated cell membrane activity.

#### 8-Br-cAMP-induced heterologous desensitization

Treatment of hepatocytes with 10  $\mu$ M 8-Br-cAMP produces what appears to be only a partial heterologous desensitization compared to that seen following glucagon treatment (Table 4-2). 8-Br-cAMP treatment produces a significant reduction in subsequent NaF-stimulated adenylyl cyclase activity, with a magnitude of only about one half the effect produced by glucagon treatment. I wondered whether this effect of 8-Br-cAMP might be somehow related to the decreased ability of desensitized membrane adenylyl cyclase to respond to stimulation by added G<sub>s</sub>. Membranes from untreated and

Figure 5-2. Effect of exogenous  $G_s$  on NaF-stimulated hepatocyte membrane adenylyl cyclase activity following glucagon treatment. Chick hepatocytes were treated with  $1\ \mu\text{M}$  glucagon for 2 h prior to homogenization. Membranes were mixed with  $12.5\ \text{nM}$  purified human erythrocyte  $G_s$  or dilution buffer and allowed to sit on ice for 10 min before addition to standard adenylyl cyclase assays. Adenylyl cyclase activity stimulated by NaF in the absence and presence of added  $G_s$  protein is shown for four different preparations of untreated (closed bars) and glucagon-treated cell membranes (hatched bars). Note that the axes are truncated for comparison. This decrease was observed in 8 independent membrane preparations, and the NaF-stimulated adenylyl cyclase activity of untreated and glucagon-treated hepatocyte membranes in the presence of  $12.5\ \text{nM}$   $G_s$  was significantly different ( $P < 0.01$ ).



8-Br-cAMP-treated hepatocytes were supplemented with purified  $G_s$  to assess its ability to restore full NaF-stimulated adenylyl cyclase activity to the treated membranes. In Figure 5-3,  $G_s$  supplementation is shown to be ineffective in eliminating the difference in activity between 8-Br-cAMP-treated and untreated hepatocyte membranes. Despite the elevation of absolute adenylyl cyclase activities due to the high concentration of added  $G_s$ , a significant difference remains between the control and 8-Br-cAMP-treated membranes. The lack of ability of exogenous  $G_s$  to correct the defect in 8-Br-cAMP-treated cell membranes suggests that the adenylyl cyclase in these membranes is less able to express  $G_s$  stimulation. This refractoriness to exogenous  $G_s$  supports a role for the cAMP-dependent protein kinase in the heterologous desensitization induced by glucagon treatment of chick hepatocytes.

#### Protein kinase A treatment of hepatocyte membranes

Since the reduction of  $G_s$  stimulation of desensitized hepatocyte membrane adenylyl cyclase appeared to cAMP-mediated, I tested whether *in vitro* treatment of hepatocyte membranes with cAMP-dependent protein kinase and ATP could have the same effect as hormone or 8-Br-cAMP treatment of intact cells. Membranes from untreated or glucagon-treated (2 h) hepatocytes were preincubated with the purified catalytic subunit of protein kinase A and ATP prior to assay for adenylyl cyclase activity. In Figure 5-4, preincubation of naive membranes with 50 nM protein kinase A and ATP significantly reduces subsequently determined NaF-stimulated adenylyl cyclase activity compared to preincubation without the kinase added. However, the protein kinase A

Figure 5-3. Effect of exogenous  $G_s$  on NaF-stimulated adenylyl cyclase activity in membranes following 8-Br-cAMP treatment of hepatocytes. Chick hepatocytes were treated with 1  $\mu$ M glucagon or with 10  $\mu$ M 8-Br-cAMP for 30 min prior to homogenization. Membranes were mixed with 12.5 nM purified human erythrocyte  $G_s$  or dilution buffer and allowed to sit on ice for 10 min before addition to standard adenylyl cyclase assays. NaF-stimulated adenylyl cyclase activity in the absence and presence of added human erythrocyte  $G_s$  is shown for untreated (closed bars), glucagon-treated (hatched bars) and 8-Br-cAMP-treated cell membranes (open bars). Note that the axes were truncated for comparison. This experiment was repeated with two independent preparations of 8-Br-cAMP-treated cell membranes.

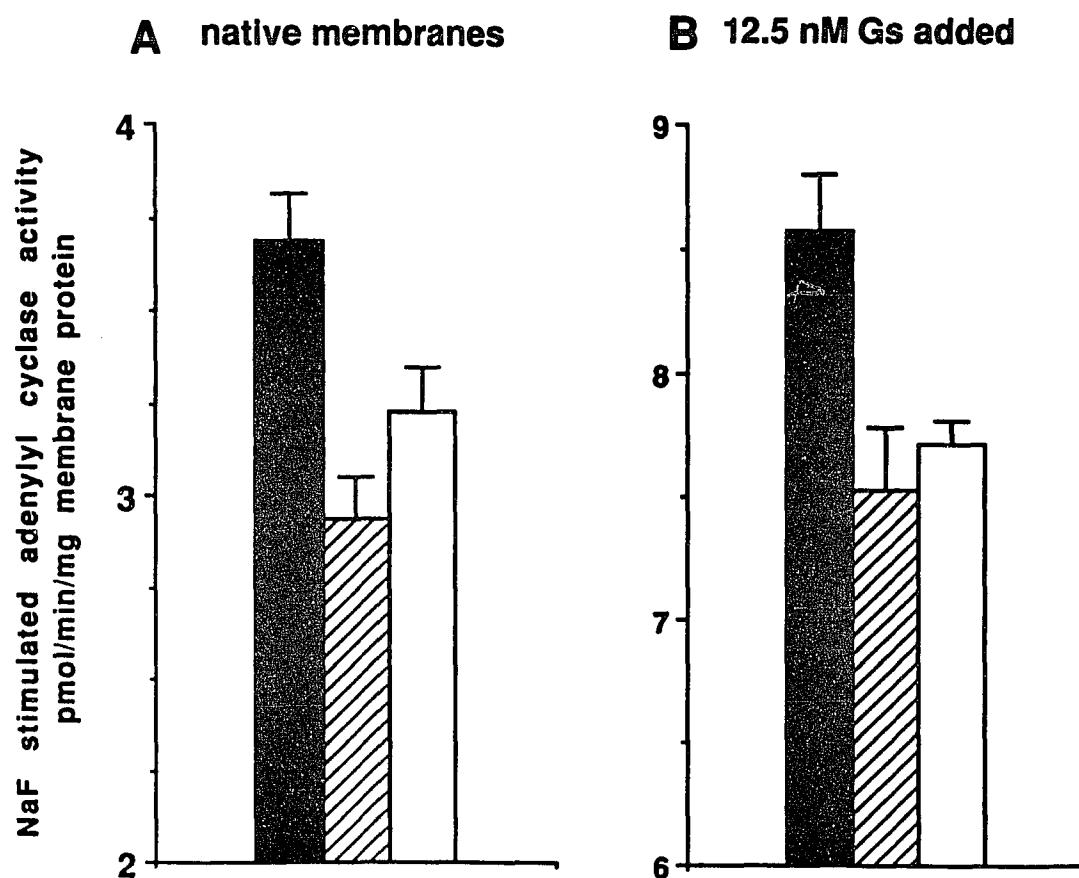
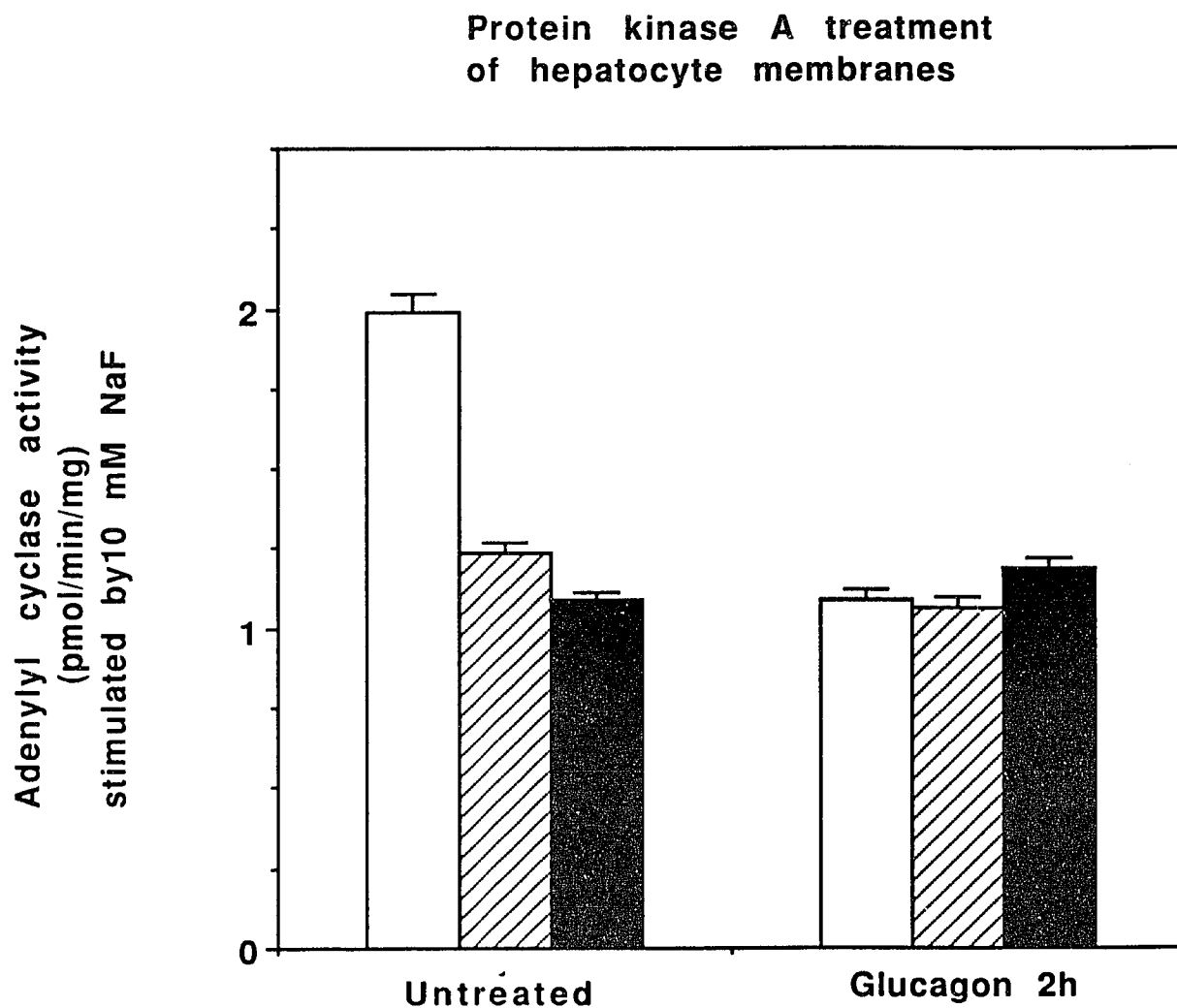


Figure 5-4. Effect of protein kinase A pretreatment on hepatocyte membrane adenylyl cyclase activity. Chick hepatocytes were treated with 1  $\mu$ M glucagon for 2 h prior to homogenization. Untreated and glucagon-treated cell membranes were incubated for 10 min at 32°C in the absence or presence of 50 nM catalytic subunit of protein kinase A purified from porcine heart. This pre-assay incubation mixture (35  $\mu$ l per final 50  $\mu$ l adenylyl cyclase assay) contained all components of the adenylyl cyclase assay except the stimulator (10 mM NaF) and the radioisotopes ( $[^3\text{H}]\text{cAMP}$  and  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ ). The ATP concentration during the kinase treatment was thus 143  $\mu$ M. The pretreated membranes (35  $\mu$ l) were then added to 15  $\mu$ l containing NaF and the radioisotope mixture, and adenylyl cyclase activity assayed by incubation at 32°C for 10 min. NaF-stimulated activity of membranes incubated without kinase is shown as the open bar. Two preparations of protein kinase A were used: one was the gift of Dr. J. D. Corbin (hatched bars) and the other was obtained through Promega (closed bars).

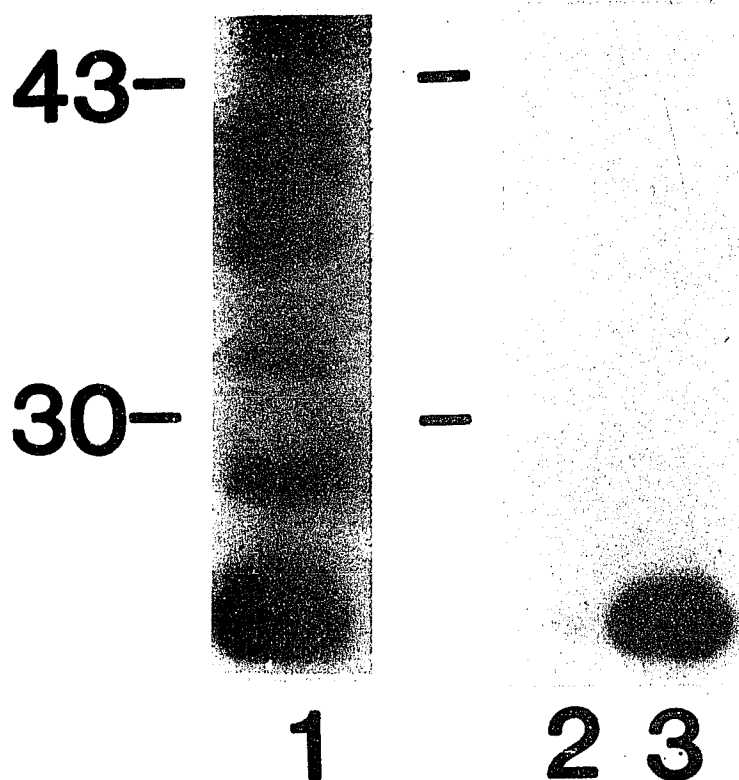


pretreatment of membranes from glucagon desensitized cells had no further desensitizing effect on NaF stimulated adenylyl cyclase activity. This lack of protein kinase A effect would be expected if the glucagon-induced heterologous desensitization involves protein kinase A phosphorylation of a target protein, since these sites would have already been phosphorylated during the glucagon treatment of the intact cell.

#### Phosphorylation of purified G proteins with protein kinase A

Since NaF stimulation of adenylyl cyclase involves only  $G_s$  and the catalytic unit of adenylyl cyclase itself (Northup *et al*, 1983a), protein kinase A phosphorylation sites regulating NaF-stimulated adenylyl cyclase activity can only reside on one or both of these proteins. The deduced amino acid sequence of  $G_s$ - $\alpha$  has no consensus protein kinase A phosphorylation sites (Robishaw *et al*, 1986a,b, Bray *et al*, 1986). To further eliminate the possibility that protein kinase A phosphorylation of  $G_s$  is responsible for the observed functional effects, the ability of purified protein kinase A to directly phosphorylate purified  $G_s$  was assessed. 5  $\mu$ g of partially purified preparations of human erythrocyte G proteins ( $G_s$ ,  $G_i$  and  $G_z$  mixture: see Premont *et al*, 1989) were incubated with 5 nM PKA catalytic subunit and [ $\gamma$ - $^{32}$ P]ATP (Figure 5-5). Under conditions where the protein kinase A extensively phosphorylated a 24 kDa contaminant protein, none of the G protein subunits ( $\alpha_s$ ,  $\alpha_i$ ,  $\alpha_z$ ,  $\beta_{35}$ , or  $\beta_{36}$ ) appeared to be phosphorylated to any significant degree. Thus, the catalyst of adenylyl cyclase, rather than  $G_s$ , appears likely to be the locus of this protein kinase A-dependent modulation of hepatocyte membrane adenylyl cyclase activity.

Figure 5-5. Phosphorylation of purified G proteins with protein kinase A. A mixture of  $G_s$ ,  $G_i$ , and  $G_z$  proteins (Premont *et al*, 1989) purified from human erythrocytes (gift of J. Codina) were incubated with 5 nM protein kinase A and [ $\gamma$ - $^{32}$ P]ATP. A Coomassie blue-stained gel of the G protein mixture is shown in lane 1. The three major protein bands at the top of the lane are (from the 43 kDa standard):  $\alpha_s$ ,  $\alpha_i$  (mainly  $G_{i3}$ ), and both  $\beta_{36}$  and  $\beta_{35}$ .  $G_z\alpha$  immunoreactivity is also found in this protein preparation at 41 kDa. The lower molecular weight bands are non-G protein contaminants. Phosphorylated G proteins were separated by SDS-PAGE and the dried gel was exposed to X-ray film. A photograph of the autoradiogram is shown. Lane 2 is a phosphorylation reaction containing just the protein kinase A (which is of approximately 40 kDa and is autophosphorylated). Lane 3 is the protein kinase A labeled G protein preparation.



### Protein kinase A treatment of S49 *kin*<sup>-</sup> cell membranes

Since the effects of protein kinase A treatment of membranes or of 8-Br-cAMP treatment of intact hepatocytes are relatively small in extent, I was concerned that the unknown constant activation of adenylyl cyclase by factors in the culture serum may be masking a larger change.

Kunkel *et al* (1989) have reported that addition of protein kinase A and ATP to *kin*<sup>-</sup> S49 mouse lymphoma cell membranes (but not *wt* S49 cell membranes) reduced forskolin-stimulated adenylyl cyclase activity by 24% when assayed in the presence of low magnesium. Since the *kin*<sup>-</sup> variant of S49 cells lack protein kinase A activity (Coffino & Tomkins, 1975; Steinberg *et al*, 1978), any protein kinase A phosphorylation sites on target proteins in these cells should be naive, allowing a full effect to be produced upon treatment of membranes with exogenous kinase. Wild-type cells contain protein kinase A, which would presumably be activated by factors in the culture media and sera which activate adenylyl cyclase, and substrate proteins would therefore have some level of phosphorylation. I was therefore interested whether the *kin*<sup>-</sup> S49 cell membranes might also exhibit protein kinase A-dependent regulation similar to that seen in chick hepatocytes.

Clark and coworkers had demonstrated that the receptor-dependent  $\beta$ -adrenergic heterologous desensitization response of S49 cell adenylyl cyclase measured in membranes varied depending on the  $Mg^{2+}$  concentration used in the adenylyl cyclase assay (Clark *et al*, 1987; Kunkel *et al*, 1989). Therefore I assayed the NaF- and forskolin-stimulated adenylyl cyclase activity of protein kinase A-treated S49 *kin*<sup>-</sup>

membranes in various  $\text{MgCl}_2$  concentrations to see if protein kinase A treatment of the membranes had any effect and to determine the optimal  $\text{MgCl}_2$  concentration for the kinase effect. Membranes from *kin*<sup>-</sup> S49 cells were preincubated with or without protein kinase A and ATP, and then tested for alteration in NaF- and forskolin-stimulated adenylyl cyclase activity. In Figure 5-6, the decrease in adenylyl cyclase activity stimulated by NaF and forskolin following treatment of the *kin*<sup>-</sup> membranes with protein kinase A is shown for one experiment versus total  $\text{MgCl}_2$  concentration in the assay. Clearly, these membranes show a protein kinase A-mediated reduction in NaF- and forskolin-stimulated adenylyl cyclase activities, with larger losses apparent at lower  $\text{MgCl}_2$  concentration in the assay.

I chose 2.5 mM  $\text{MgCl}_2$  as the optimal magnesium concentration, since lower divalent ion concentrations produce a larger apparent protein kinase A-mediated desensitization but also less stimulated adenylyl cyclase activity. This concentration represents a balance between these two considerations. Table 5-1 summarizes the results of many similar *kin*<sup>-</sup> membrane/protein kinase A treatment experiments under these conditions. Both NaF- and forskolin-stimulated adenylyl cyclase activities in protein kinase A-pretreated S49 *kin*<sup>-</sup> cell membranes appeared to be significantly reduced compared with untreated membranes, by approximately 20%. Basal activity was too low compared with background of the assay to determine whether it was also affected. The ability of protein kinase A treatment of both hepatocyte and S49 cell membranes to produce a similar effect on adenylyl cyclase activity implies that the cAMP-dependent effect observed in the hepatocytes may be a more generally-occurring phenomenon.

Figure 5-6. Effect of protein kinase A pretreatment on S49 *kin* lymphoma cell membrane adenylyl cyclase activity assayed at varying magnesium concentrations. Membranes were prepared from untreated *kin* S49 lymphoma cells. Membranes were incubated in the presence of 50 nM protein kinase A catalytic subunit and 143  $\mu$ M ATP for 10 min at 32°C, in the presence of all components of the adenylyl cyclase assay except stimulators, [ $^3$ H]cAMP and [ $\alpha$ - $^{32}$ P]ATP. After 10 min, the membrane mixture was removed to ice and immediately assayed in triplicate for adenylyl cyclase activity by addition of 35  $\mu$ l of the membrane treatment mix to 15  $\mu$ l of stimulator and radioisotopes. The final ATP concentration in the adenylyl cyclase assay was 100  $\mu$ M. Adenylyl cyclase activity stimulated by NaF (open circles) and forskolin (closed circles) is shown as the percentage of activity measured in protein kinase-treated membranes compared to untreated membranes (mean  $\pm$  SD for triplicate determinations) versus the total  $\text{MgCl}_2$  concentration in each final adenylyl cyclase assay.

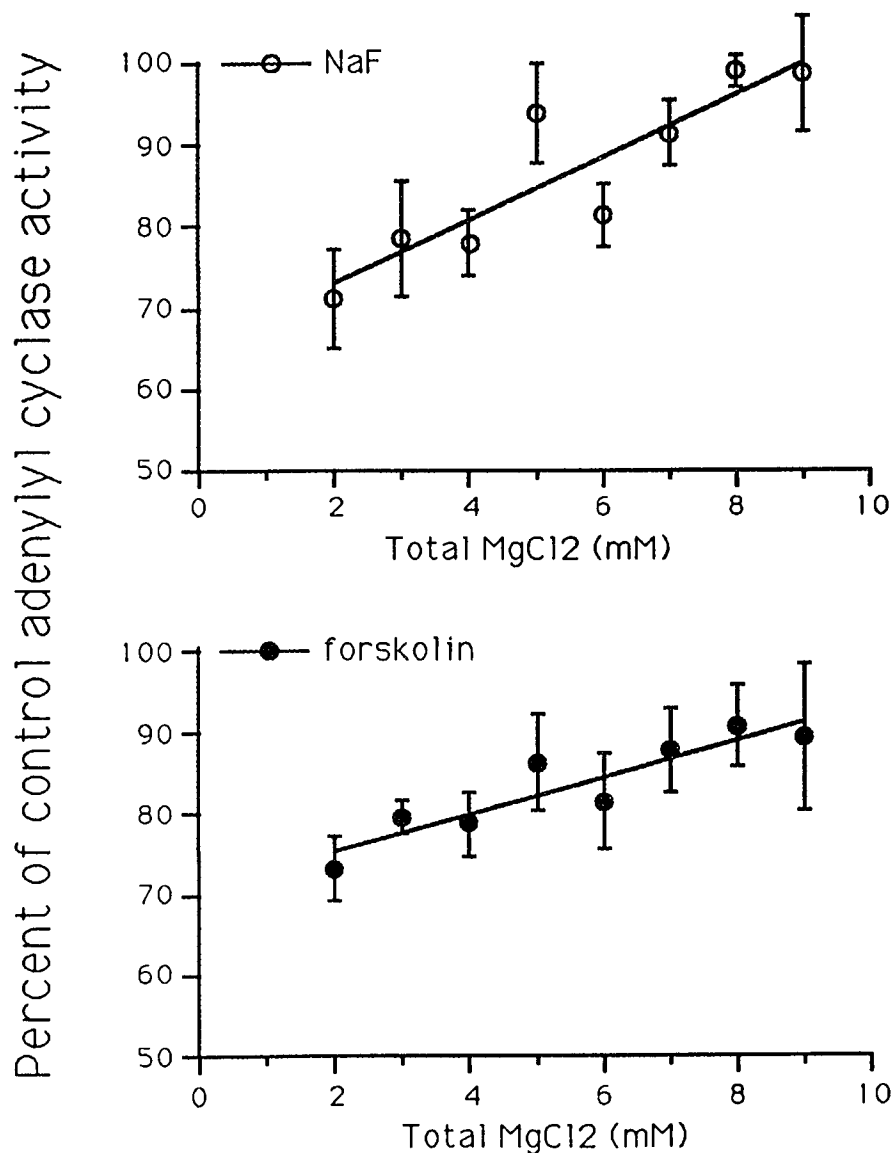


Table 5-1. Protein kinase A pretreatment of *kin*<sup>-</sup> S49 cell membranes. Membranes were prepared from untreated *kin*<sup>-</sup> S49 cells. Membranes were pretreated in the absence and presence of 50 nM protein kinase A and 143  $\mu$ M ATP for 10 min at 32°C in the presence of components of the adenylyl cyclase assay (except stimulators, [<sup>3</sup>H]cAMP and [ $\alpha$ -<sup>32</sup>P]ATP). After 10 min, the membrane mixture was removed to ice and immediately assayed in triplicate for adenylyl cyclase activity by addition of 35  $\mu$ l of the membrane treatment mix to 15  $\mu$ l of stimulator and radioisotopes. The total MgCl<sub>2</sub> concentration in the final assay was 2.5 mM, and ATP was 100  $\mu$ M. This experiment was repeated 10 times using three different batches of S49 cell membranes. Data are shown as cumulative mean  $\pm$  SEM of the percent decrease in stimulated adenylyl cyclase activity (compared to untreated membranes) following protein kinase A treatment. NaF- and forskolin-stimulated adenylyl cyclase activities are significantly reduced ( $p < 0.005$ ) following treatment of membranes by protein kinase A.

| Stimulator           | Percent decrease in<br>adenylyl cyclase activity<br>compared to untreated membranes |
|----------------------|-------------------------------------------------------------------------------------|
| <div></div>          |                                                                                     |
| basal (none)         | 85.1 $\pm$ 17.3                                                                     |
| 10 mM NaF            | 76.9 $\pm$ 9.4                                                                      |
| 10 $\mu$ M forskolin | 80.5 $\pm$ 8.5                                                                      |

### Summary

It is well established that receptor-mediated stimulation of adenylyl cyclase requires only three components: the receptor,  $G_s$ , and adenylyl cyclase (May *et al*, 1985; Feder *et al*, 1985). Heterologous desensitization is a functional description used to describe all modifications of the adenylyl cyclase system which reduce its ability to be stimulated by hormones acting through receptors other than that used to induce the desensitization. Alterations of any of these three components of the adenylyl cyclase system may result in a heterologous desensitization. Previous studies of heterologous desensitization of adenylyl cyclase have suggested cAMP-dependent phosphorylation of hormone receptors (Sibley *et al*, 1984; Benovic *et al*, 1985; Clark *et al*, 1988) or modulation of  $G_s$  (Kassis & Fishman, 1982; Kirchick *et al*, 1983; Garrity *et al*, 1983; Premont & Iyengar, 1989) as the basis for regulation. I have presented evidence here that adenylyl cyclase itself may be another locus of regulation. Each of these effects alone is rather small in extent. Nevertheless, the cumulative effect of these reductions allows the hepatocyte to lower its sensitivity to hormone stimulation of adenylyl cyclase.

Heterologous desensitization of the chick hepatocyte adenylyl cyclase system induced by glucagon appears to involve two of these three possible types of alterations.  $G_s$  activity, as determined by reconstitution of *cyc*<sup>-</sup> S49 cell membrane adenylyl cyclase, is reduced in glucagon-treated hepatocyte membranes, indicating that modulation at the level of G proteins plays a role in the heterologous desensitization (Chapter 4). If this decrease in  $G_s$  activity were the sole cause of the heterologous desensitization, addition of exogenous  $G_s$  would be expected to restore NaF-stimulated adenylyl cyclase activity

of glucagon-treated cell membranes to levels comparable to that measured in membranes from cells which had never seen glucagon. However, this does not appear to be the case. Membranes from glucagon-treated hepatocytes express lower NaF-stimulated adenylyl cyclase activity than membranes from untreated cells following addition of purified  $G_s$ , even as both activities are increased at higher  $G_s$  concentrations. Thus, heterologous desensitization also involves a reduction in the ability of adenylyl cyclase in glucagon-treated membranes to respond to stimulation by exogenous  $G_s$ .

In many systems, heterologous desensitization occurs *via* phosphorylation of heterologous hormone receptors by protein kinase A, leading to their uncoupling from  $G_s$  (Clark *et al*, 1988; Benovic *et al*, 1985). Glucagon appears to induce this form of heterologous desensitization of MDCK cell adenylyl cyclase. When glucagon-treated MDCK cell membranes were supplemented with exogenous  $G_s$ , NaF stimulation of adenylyl cyclase was restored to untreated levels, but hormonal ( $PGE_1$ ) stimulation remained desensitized (Rich *et al*, 1984). Thus a functional block at the level of the  $PGE_1$  receptor coupling to  $G_s$  was present in the desensitized membranes, and is most probably due to protein kinase A phosphorylation of the receptors. In the chick hepatocyte system, stimulation of adenylyl cyclase by both NaF and a heterologous hormone (VIP) were both restored to equivalent extents by addition of purified  $G_s$  to desensitized membranes, indicating that this receptor-level regulation is not an important component of heterologous desensitization in chick hepatocytes, at least for the VIP receptor.

Glucagon- or 8-Br-cAMP-treated hepatocyte membrane adenylyl cyclase appears less able to respond to stimulation by added NaF/ $G_s$  than control membrane adenylyl cyclase. From the  $G_s$  supplementation assays, it is clear that adenylyl cyclase is not the limiting component for NaF-stimulated activity in the membrane, since addition of sufficient "extra"  $G_s$  can result in a doubling of the observed adenylyl cyclase activity. Since NaF/ $G_s$  directly activates adenylyl cyclase (Northup *et al*, 1983a), during desensitization some modification event reduces the capability of the catalyst to function as a linear readout system for the activity state of  $G_s$ . Three possibilities may account for this change: the catalytic rate of the enzyme may be lowered; the catalyst may be altered so that it is less responsive to  $G_s$ ; or desensitized hepatocyte membranes may contain fewer catalytic subunits than do untreated membranes. This phase of heterologous desensitization does not require a physical loss of the catalyst of adenylyl cyclase since this effect can be mimicked by treatment of membranes from both chick hepatocytes and *kin* S49 cells with protein kinase A and ATP. Since activity stimulated by both NaF and forskolin are reduced following protein kinase A treatment of S49 cell membranes, it might seem that the reduction in the catalytic rate of the adenylyl cyclase is the clear mechanism for the protein kinase A effect. However, while forskolin can stimulate adenylyl cyclase directly, it stimulates much more extensively in the presence of  $G_s$  (Seamon & Daly, 1981a; Seamon *et al*, 1981). Since forskolin stimulation is sensitive to alterations in  $G_s$ -adenylyl cyclase coupling, changes in forskolin-stimulated adenylyl cyclase activity are not informative as to whether the kinase treatment affects catalytic activity directly or affects activity by altering  $G_s$  coupling to adenylyl cyclase. (In

hepatocytes, the small amount of glucagon remaining on the membranes from treated cells synergistically activates adenylyl cyclase in the presence of forskolin. Since it is impossible to predict how much glucagon remains or to strip it all away in washing without affecting the activity of the membrane, loss of forskolin stimulation could not be reliably measured in hepatocyte membranes).

Alteration of the catalyst of adenylyl cyclase by direct covalent modification, such as by phosphorylation, appears the simplest explanation for these data. 8-Br-cAMP treatment of hepatocytes or protein kinase A treatment of membranes produce similar reductions in NaF-stimulated activity of adenylyl cyclase. Concurrent regulation and phosphorylation of adenylyl cyclase in frog erythrocytes treated with phorbol esters indicates that adenylyl cyclase activity can be modulated by its protein phosphorylation state (Yoshimasa *et al*, 1987). Indeed, Yoshimasa *et al* (1988) reported that an adenylyl cyclase purified from bovine striatum served as a substrate for protein kinase A, and that phosphorylation reduced  $G_s$ -stimulated activity of adenylyl cyclase in a reconstituted system. In the rat reticulocyte system, Yamashita *et al* (1989) found that a similar isoproterenol- (and cAMP-) induced heterologous desensitization is associated with an apparent loss in the stability of the  $G_s$ -adenylyl cyclase complex. A decrease in the affinity of adenylyl cyclase for activated  $G_s$ , rather than a decreased catalytic rate or loss of adenylyl cyclase protein, is my tentative mechanistic explanation for this component of heterologous desensitization.

Confirmation of this proposed negative feedback regulation of adenylyl cyclase by direct protein kinase A phosphorylation of adenylyl cyclase will require three types

of experiments: demonstration that the adenylyl cyclase protein can be phosphorylated by the kinase, demonstration that the protein is phosphorylated in the cell in response to an appropriate hormonal signal, and demonstration that the activity of the enzyme is regulated by the phosphorylation event. Purification of adenylyl cyclase from liver using forskolin affinity chromatography yielded an apparently homogeneous preparation of the enzyme with a 135 kDa size and estimated turnover number of 10,000/min for  $\text{MnCl}_2$  and forskolin stimulation. However, it was not feasible to enhance the recovery enough to obtain sufficient amounts of the protein to undertake studies to examine its phosphorylation *in vitro* by protein kinase A, and determine the effects of this phosphorylation on activity. Attempts to immunoprecipitate the S49 cell adenylyl cyclase using an anti-calmodulin-sensitive adenylyl cyclase monoclonal antibody in order to determine the phosphorylation state of the protein have also been unsuccessful. Instead, I have examined the adenylyl cyclase in liver by cloning cDNAs encoding *two* novel forms of the enzyme which form a distinct subfamily of mammalian hormone-stimulated adenylyl cyclases.

## CHAPTER 6

### STRUCTURE OF LIVER ADENYLYL CYCLASES

In order to better understand the molecular basis for functional changes in adenylyl cyclase activity during desensitization, such as by phosphorylation, I set out to determine the primary structure of liver adenylyl cyclase. I had initially hoped to obtain sufficient purified enzyme from porcine liver to obtain direct amino acid sequence from proteolytic peptide fragments, but the purification suffered from low yield. However, in the summer of 1989, two groups reported the cloning of bovine brain adenylyl cyclase cDNAs, and I set out to clone the cDNA for the liver adenylyl cyclase by homology screening.

Utilizing the forskolin affinity chromatography developed by Pfeuffer and coworkers (Pfeuffer & Metzger, 1982; Pfeuffer, 1991), Krupinski *et al* (1989) purified a 110 kDa  $G_s$ - and calmodulin-stimulated adenylyl cyclase from bovine cortex (Smigel, 1986), and determined the amino acid sequence of several proteolytic fragments which were used to design oligonucleotide probes cloning for the calmodulin-sensitive adenylyl cyclase cDNA. Expression of the cloned sequence was demonstrated to yield increased forskolin and hormone-stimulated adenylyl cyclase activity. Lipkin *et al* (1989a,b) raised monoclonal antibodies against a partially-purified bovine brain adenylyl cyclase preparation and selected one which inhibited calmodulin-insensitive adenylyl cyclase. They purified a 130 kDa protein from bovine brain on an immunoaffinity column, and

cloned its cDNA using oligonucleotide probes based on the sequence of proteolytic fragments of the purified protein. No expression was attempted. These two sequences share no similarity in sequence or overall topology.

The sequence of the liver adenylyl cyclase mRNA would be of great value in understanding the structural basis for functional changes in adenylyl cyclase activity during desensitization. Sites of protein kinase A phosphorylation can be identified and then analyzed through mutation and expression of the cDNA. Further, many basic issues of how adenylyl cyclase catalyzes cAMP formation (ATP binding site structure, why the enzyme is not product-inhibited) as well as how it is regulated ( $G_s$  binding site,  $G_i$  binding site in inhibitable isoforms, forskolin binding site) have simply not yet been addressed. In order to isolate the cDNA encoding liver (calmodulin-insensitive) adenylyl cyclase, I recloned both of the recently reported bovine adenylyl cyclase cDNAs from a bovine brain cDNA library for use as probes for the liver form of the enzyme. Using the calmodulin-sensitive adenylyl cyclase as a probe, I have identified and isolated two distinct adenylyl cyclase-related sequences from rat liver which are shown to encode two members of a new subfamily of adenylyl cyclases.

#### Cloning of bovine brain calmodulin-sensitive adenylyl cyclase type I

A size-selected (2→4.4kb and >4.4kb fractions) bovine brain cDNA library in  $\lambda$ ZAP II was initially screened at 45°C with a mixture of three 50-mer oligonucleotides complementary to the sequence of bovine brain calmodulin-sensitive (type 1) adenylyl cyclase (corresponding to amino acids 38-51 [probe 13], 324-340 [probe 12], and 990-

1006 [probe 11] in Krupinski *et al* (1989)). Since this same library had been used for the original cloning and no full-length insert was found, I expected no clones to hybridize positively to all three probes. Initial positive phage were categorized for insert size and probe reactivity by a PCR-Southern hybridization technique as follows. Putative positive phage stocks were amplified by polymerase chain reaction with Taq DNA polymerase using the T7 and T3 promoter 17-mer sequencing primers as PCR primers. The amplified product bands thus were full-size copies of all inserts present in the phage mixture. The PCR reaction was electrophoresed on a 1% agarose gel and transferred by capillary action to Nytran nylon filters. The filters were then tested for reactive inserts by hybridization with only a single type 1 adenylyl cyclase probe at a time. Phage could then easily be sorted by insert size and probe reactivity while quite impure, and duplicates eliminated. One positive phage, containing a 2.7 kb insert and reacting with amino-terminal probe 13, was purified to homogeneity and subcloned by phagemid excision. This clone (13[2]-10.1) corresponded to clone pA22 of Krupinski *et al* (1989), which extends from base 107 to 2570, covering 60% of the coding region. Clones encoding the carboxyl terminal portion of brain adenylyl cyclase were also sorted in the same manner, but have not yet been further purified.

At the UCLA Symposium on "G Proteins" in February 1990, H. Bakalyar and R. Reed reported the cloning of an olfactory neuron-specific isoform of adenylyl cyclase by low-stringency screening with the brain adenylyl cyclase clone. This new form of adenylyl cyclase (called type 3) was 28% identical overall with the brain type I clone, but had much higher identity concentrated in those regions of the molecule encoding the

duplicated, presumed-intracellular loops. In his Harvey Lecture shortly thereafter, A. G. Gilman reported the cloning of further brain and testes forms of adenylyl cyclases (types 2 and 4) which also had high identity with these other adenylyl cyclases concentrated in the intracellular loop regions. The proposed membrane spanning regions appear less highly conserved. The missing portion of clone 13[2]10.1 contained one of these regions of high identity. At this time, it seemed clear that this region would be the best probe to use for the low-stringency screening of a liver cDNA library to identify liver adenylyl cyclase clones. To more rapidly obtain just such a cloned DNA fragment, aliquots of the >4.4 kb  $\lambda$ ZAP library were subjected to PCR amplification from oligonucleotide primers spanning the desired sequence in the type 1 adenylyl cyclase. Two such reactions were performed, yielding expected 1462 bp (primer 21→25) and 921 bp (primer 20→25) amplified products, from the last membrane span or from the beginning of the high homology region to the stop codon. These PCR products were subcloned into pBS, sequenced, and excised for use as hybridization probes.

#### Cloning of bovine brain "calmodulin-insensitive adenylyl cyclase"

Two oligonucleotides, complementary to residues 1-36 of clone pAC111 (5'-GCCCTCCATGCTGGCCTCCTTGGCATCATACCACTT) and to the reverse translated product of peptide III.2 (5'-GTGCCAGACCTCCTCGCCCATGGCICGCCACTCGGC) in Lipkin *et al* (1989a), were prepared. These probes were end labelled by 5' phosphorylation using polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP, and used to screen 2→4.4kb and >4.4kb size-selected bovine brain cDNA libraries in  $\lambda$ ZAPII at 45°C. Three

independent phage were identified from  $4 \times 10^5$  phage screened from the >4.4kb size fraction library, all of which were full-length. Two additional clones (one full-length) were isolated from the 2→4.4kb library. Sequencing reveals that these clones correspond to the sequence in Lipkin *et al* (1989b) or are splice variants of that sequence.

A comparison of these nucleotide and amino acid sequences against the GenBank (release 68.0) and GenPept (release 64.3) databases (Bilofsky & Burks, 1988) using the FASTA program of Pearson & Lipman (1988) revealed that they have very high identity with the products of the neural cell adhesion molecule (N-CAM) gene family, *but not* adenylyl cyclase (Premont, 1991). These sequences are clearly the bovine versions of the N-CAM gene products, as the deduced amino acid sequence has 94% identity with rat and human, and 93% with mouse N-CAM forms (Figure 6-1). N-CAM is known to exist in three major splicing classes, encoding proteins of 110, 130 and 160 kDa, with many minor splice variants within each class (Cunningham *et al*, 1987). Both the 130 and 160 kDa forms pass through the membrane a single time, while the 110 kDa form is tethered to the cell membrane by a phosphatidyl inositol anchor. Thus clone 4-1.1, the most extensively characterized, encodes the 130 kDa form of bovine brain N-CAM, as does the sequence reported by Lipkin's group (Lipkin *et al*, 1989a,b). Other clones encode the other major splice variants. The low homology reported between this sequence and yeast adenylyl cyclases (Lipkin, 1989a,b) was judged insignificant upon comparison to known (expressed) mammalian adenylyl cyclases (Premont, 1991). Since these "adenylyl cyclase" N-CAM clones do not appear to encode an adenylyl cyclase, I have not used these sequences to search for related (non-adenylyl cyclase) cDNAs from the liver.

Figure 6-1. Comparison of the deduced amino acid sequence of "calmodulin-insensitive adenylyl cyclase" from Lipkin *et al* (1989b) (BOVADCYC, GENBANK accession number X16451) with a rat N-CAM sequence. The FASTA program (Pearson & Lipman, 1988) was used with a ktup value of 1 to search the GenPept database (release 64.3) for similarities to the BOVADCYC deduced amino acid sequence. An alignment produced by FASTA with the rat N-CAM sequence RATNCAM14 (accession number X06564), which had the highest overall identity, is shown using one letter amino acid code. Identical residues are indicated with ":" and conservative substitutions with "."; gaps inserted to maintain alignment are indicated with "-".

```

BOVADCYC  MLQTKNLIWTLFFLGTA VSLQVDIVPSQGEISVGESKFFLCQVAGDAKDKDISWFS PNGE  60
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  MLRTKDLIWTLEFFLGTA VSLQVDIVPSQGEISVGESKFFLCQVAGDAKDKDISWFS PNGE  60

BOVADCYC  KLTPNQQRISVVWNDDSSSTLT IYNNANIDDAGIYKCVVTAEDGTESEATVNVKIFQKLMF 120
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  KLSPNQQRISVVWNDDSSSTLT IYNNANIDDAGIYKCVVTAEDGTQSEATVNVKIFQKLMF 120

BOVADCYC  KNAPTPQEFREGEDAVIVCDVVS LPP TTIWKHKGRDVILKKDVR FIVLTNNYLQIRGIK 180
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  KNAPTPQEFKEGEDAVIVCDVVS LPP TTIWKHKGRDVILKKDVR FIVLSNNYLQIRGIK 180

BOVADCYC  KTDEGTYRCEGRILARGEINFKDIQVIVNVPPTVQARQSI V NATANLGQSVTLVCNAEGF 240
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  KTDEGTYRCEGRILARGEINFKDIQVIVNVPPTVQARQSI V NATANLGQSVTLVCDADGF 240

BOVADCYC  PEPTVSWTKDGEQIENEE--DEKYLFSDDSSSEL TIRKVDKNDEAEYVCIAENKAGEQDAS 298
           :: :::::::::: :::: :::::::::::::::::: :::::::::::::::::: ::::::::::
RATNCAM14  PEPTMSWTKDGEPIENEEEDDEKHIFSDDSSSEL TIRNV DKNDEAEYVCIAENKAGEQDAS 300

BOVADCYC  IHLKVFAKPKITYVENQTAMELEE QVTLTCEASGDPIPSITWRTSTRNISSE EKASWTRP 358
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  IHLKVFAKPKITYVENQTAMELEE QVTLTCEASGDPIPSITWRTSTRNISSE EKASWTRP 360

BOVADCYC  EKQETLDGHMVVRSHARVSS LTLKSIQYTDAGEYVCTASNTIGQDSQSMYLEVQYAPKLQ 418
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  EKQETLDGHMVVRSHARVSS LTLKSIQYTDAGEYICTASNTIGQDSQSMYLEVQYAPKLQ 420

BOVADCYC  GPVAVYTWEQNQVNITCEVFAYPSATISWFRD GQLLPSSNYSNIKIYNTPSASYLEVTPD 478
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  GPVAVYTWEQNQVNITCEVFAYPSATISWFRD GQLLPSSNYSNIKIYNTPSASYLEVTPD 480

BOVADCYC  SENDFGNYNCTAVNRIGQESLEFVLVQADTPSSPSIDQVEPYSS TAQVQFDEPEATGGVP 538
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  SENDFGNYNCTAVNRIGQESLEFILVQADTPSSPSIDRVEPYSS TAQVQFDEPEATGGVP 540

BOVADCYC  ILKYKAEWRAMGEEVWHKWDAKEASMEGIVTIVGLKPETTYAVRLAALNGKGLGEISA 598
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  ILKYKAEWKS LGEEAWH KWDAKEANMEGIVTIMGLKPETRYAVRLAALNGKGLGEISA 600

BOVADCYC  ASEFKTQPVREPSAPKLEGQMGEDGNSIKVKLIKQDDGGSP I RHYLVKYRALSSEWKPEI 658
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  ATEFKTQPVREPSAPKLEGQMGEDGNSIKVNLIKQDDGGSP I RHYLVKYRALASEWKPEI 660

BOVADCYC  RLP SGSDHVMLKSLDWN AEYEVYVVAENQQGKSAAHFV FRTSAQPTAIPANGSPTAGLS 718
           :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RATNCAM14  RLP SGSDHVMLKSLDWN AEYEVYVVAENQQGKSAAHF G FRTSAQPTAIPANGSPTAGLS 720

BOVADCYC  TGAIVGILVVT FVLLLVAVDVTCYFLNKCGLLMCIAVNL CGKAGPGAKGKDMEEGKAAFS 778
           :: :::::::::: :::: :::::::::::::::::: :::::::::::::::::: ::::::::::
RATNCAM14  TGAIVGILIVIFVLLLVVMDITCYFLNKCGLLMCIAVNL CGKAGPGAKGKDMEEGKAAFS 780

BOVADCYC  KDESKEPIVEVRTEEERTPNHDG GKHTEPNETT PLTEPEKGPVEAKP---ETETKPAPAE 835
           :: :::::::::::::::::::::::::::::::::: :::::::::: :::::::::: ::::
RATNCAM14  KDESKEPIVEVRTEEERTPNHDG GKHTEPNETT PLTEPEKGPVETKSEPQESEAKPAPTE 840

BOVADCYC  VQTVPN DATQIKVNESKA 853
           : :::::::::: : ::::
RATNCAM14  VKTVPN EATQTKENESKA 858

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### Cloning of rat liver adenylyl cyclase: initial screening

A rat liver cDNA library in  $\lambda$ ZAP II was purchased from Stratagene. The library was initially probed at low stringency (45°C) using the 900 bp Xba1-Xho1 fragment of AC/1-[20→25], the PCR-amplified fragment of bovine calmodulin-sensitive (type 1) adenylyl cyclase which encodes the conserved second intracellular domain of the enzyme. Of four positive clones identified from among  $1.2 \times 10^6$  phage, all contained identical 1.4 kb inserts with an internal EcoR1 site (clone 10.1). The sequence of the 600 bp EcoR1 fragment shares 60% identity with the type 1 adenylyl cyclase within the region used as the probe and best matches that sequence among all sequences in GenBank (not shown). The 800 bp EcoR1 fragment has continued similarity to the type 1 adenylyl cyclase on the end which adjacent to the internal restriction site. However, the sequence of the opposite end has no similarity to the type 1 adenylyl cyclase or any other sequence in GenBank.

The 600 bp EcoR1 fragment of clone 10.1 was purified and used to rescreen the rat liver  $\lambda$ ZAP library at medium stringency (50°C). Of 7 phage identified in a screen of  $6 \times 10^5$  phage, five new clones were isolated. Three clones overlapped the sequence of the 600bp EcoR1 fragment of clone 10.1 and a portion of the 800 bp fragment. These overlapping clones (10.1, 26.1, 28.2, 34.1) encode sequences reaching from a position equivalent to the beginning of the first intracellular repeated domain of the brain type 1 adenylyl cyclase to a poly(A) tail at the end of a 650 bp 3' untranslated region, and included an internal EcoR1 site at the position where the 600 and 800 bp fragments of

clone 10.1 were joined. The aberrant sequence in the 800 bp fragment appears to be due to the cDNA being synthesized using an incompletely spliced RNA as template.

The remaining two new clones (27.1, 36.4) had overlapping sequence which was nearly identical but distinct from that of the first class of clones, and was also highly similar to the brain adenylyl cyclase. Thus there are in fact *two* distinct forms of adenylyl cyclase represented in the rat liver cDNA library. The original sequence I have tentatively called the type A (or type 6) and the second sequence the type B (or type 5) rat adenylyl cyclase.

The 5'-most portion of the new type 6 sequence encodes most of the first conserved cytoplasmic region. This 500 bp EcoR1 fragment of clone 28.2 was purified from an agarose gel, radiolabeled and used to rescreen the already-probed  $1.8 \times 10^6$  phage, as well as to screen another  $2.4 \times 10^6$  phage from the rat liver  $\lambda$ ZAP library. Two new positive phage were identified, which both corresponded to the type 5 form of adenylyl cyclase. Clone 45.1 contains a 3.4 kb insert, from within the first set of 6 transmembrane spans to nearly the stop codon, while clone 16.4 contains a 1.5 kb fragment of the type 5 adenylyl cyclase. Several additional screens, totaling over  $10^7$  phage, using various cDNA and oligonucleotide probes, identified only a single new clone (52.2) which contains a 1.1 kb fragment of the type 5 adenylyl cyclase. No additional 5' end sequences were identified.

A second rat liver cDNA library in  $\lambda$ gt11 was obtained from Clontech, and was screened using the full 2.2 kb clone 28.2 insert sequence. No positive clones were obtained from a screen of  $1.8 \times 10^6$  plaques. Phage were rescreened using 35-mer

oligonucleotide probes to a 5' region common to the types 5 and 6 adenylyl cyclases, again without success.

At this point, having screened several million phage from two commercial cDNA libraries and still not obtaining a 5' end for either clone, I attempted to construct specifically-primed cDNA libraries in order to obtain the missing portions of these adenylyl cyclase clones. These cDNA libraries were screened separately without prior amplification using the AC/3 28.2 500 bp EcoR1 fragment as a probe at 50°C. The 89-primed library yielded several hundred apparently positive plaques, which upon isolation contained unrelated cDNA insert sequences. The 79-primed library yielded no positive clones. The libraries were then rescreened using two 35-mer oligonucleotide probes which do not contain any sequences from the primer 89, as does the 500 bp restriction fragment used previously. No positive clones were isolated. This failure of the library may be due to structural constraint (high G+C content) of the 5' ends of the adenylyl cyclase mRNAs (seen for the other members of this family) which slows or inhibits cDNA elongation by the reverse transcriptase and leads to prematurely terminated cDNAs. Such partial clones would be selected against by the cDNA size fractionation step prior to ligation into the  $\lambda$ ZAP vector.

#### PCR rapid amplification of cDNA ends (RACE) for adenylyl cyclase 5' ends

A PCR method was used to attempt to isolate the missing 5' ends of the two liver adenylyl cyclase cDNAs using a modification of the procedure of Frohman *et al* (1988) (essentially the same as that in Loh, 1991) for the amplification of specifically-primed

first-strand cDNAs, or rapid amplification of cDNA ends (RACE). To amplify and clone the 5' ends of the two rat liver adenylyl cyclases, rat liver poly(A) RNA (1 µg) was reverse transcribed from several antisense oligonucleotide primers in separate reactions using MMLV reverse transcriptase in the presence of 10 µCi [ $\alpha$ -<sup>32</sup>P]dCTP. These primers were chosen to bind to both forms of liver adenylyl cyclase mRNA near the 5' end of the clones previously obtained. The radiolabeled cDNA-RNA hybrids were digested with mung bean nuclease to obtain blunt ends, and purified from excess primers and nucleotides by spun column chromatography on Bio-Gel 30. A pair of overlapping oligonucleotides were prepared as shown:

|           |                                       |
|-----------|---------------------------------------|
| primer 45 | 5' -CATTGAGACCGATGCCACTCGAGGC-3'      |
| primer 46 | 3' -CAAGCGTAAGTCTGGCTACGGTGAGCTCCG-5' |

Primer 46 was 5'-phosphorylated by treatment with ATP and T4 polynucleotide kinase. The phosphorylated oligonucleotide was mixed with unphosphorylated oligonucleotide 45, heated and annealed by cooling. The annealed PCR/linker (1 nmol) was mixed with the blunt-ended first-strand cDNA/RNA hybrids and ligated overnight at 16°C with T4 DNA ligase. Since the original cDNA primers were not phosphorylated, this PCR/linker can only ligate to the 3' end of the cDNA (corresponding to the 5' end of the mRNA). The RNA in the hybrids was digested with RNase A following heat-denaturation, and the resulting ligated cDNAs purified from excess linkers by passage through a spun column of Sephacryl S-300. A portion of this purified cDNA mixture (3-400 pg) was then amplified by PCR using a common antisense adenylyl cyclase primer (#89) and the PCR/linker primer 45. Products were separated on 1% agarose gels and transferred to

nitrocellulose for blotting with oligonucleotide probes. Products displaying positive hybridization signals were observed, but were found to be of only small size insufficient to even reach the end of the already-known sequences. No longer products were observed in several experiments.

#### Nested PCR screening of cDNA libraries

A second method for PCR amplification of the missing adenylyl cyclase 5' end sequences was then developed. Rather than preparing cDNA and attempting to put a known sequence on the end of the first-strand cDNA using ligation or terminal transferase (Frohman *et al*, 1988; Loh, 1991), I realized that this has effectively already been done in the construction of any cDNA library. Therefore several rat liver, kidney and heart cDNA libraries in  $\lambda$  phage vectors were screened by PCR by taking advantage of the known vector sequences at either end of every insert in the library. PCR screening was accomplished in two stages, as outlined in Figure 6-2. First, a PCR reaction using one of two vector sequence primers and an antisense adenylyl cyclase sequence primer was performed using 1  $\mu$ l of  $\lambda$  phage ( $10^6$ - $10^7$  phage) as template. Second, 1  $\mu$ l of this reaction mixture was used as template in a second PCR reaction, using the same vector primer and a second, nested antisense adenylyl cyclase sequence primer. The use of two reactions with distinct adenylyl cyclase sequence primers retains specificity otherwise lost by the use of a vector primer which matches all viruses in the reaction mix. This method is similar to that recently described by Gibbons *et al* (1991). One-tenth of the second stage reactions were run on agarose gels and transferred to nitrocellulose membranes for

Figure 6-2. Nested PCR screening of cDNA libraries. Oligonucleotide primers (17-mers) which flank the vector multiple cloning site were prepared for each vector used (V1 and V2). From the known sequence of the type 5 or 6 adenylyl cyclase, two antisense primers (A and B) were synthesized (the more 5' primer (B) also contains an EcoR1 site). Two initial reactions were performed with the 3'-most adenylyl cyclase primer (A) and each of the vector primers (V1 and V2) (Step 1, showing only V1 reaction). Each reaction yields copies of each clone in the library which is in the proper orientation relative to the vector primer, along with many (often major) non-specific products. One  $\mu$ l of these reactions is used as template for secondary reactions using the 5'-more adenylyl cyclase primer (B) and the same vector primers (Step 2, again for only V1). Those DNAs containing the primer B sequence are amplified again, effectively reducing the level of non-specific DNAs. Specifically-amplified products can be identified by hybridization to a third nested oligonucleotide probe (primer C), and the DNA band isolated and subcloned using EcoR1 sites in primer B and amplified from the vector-insert junction.

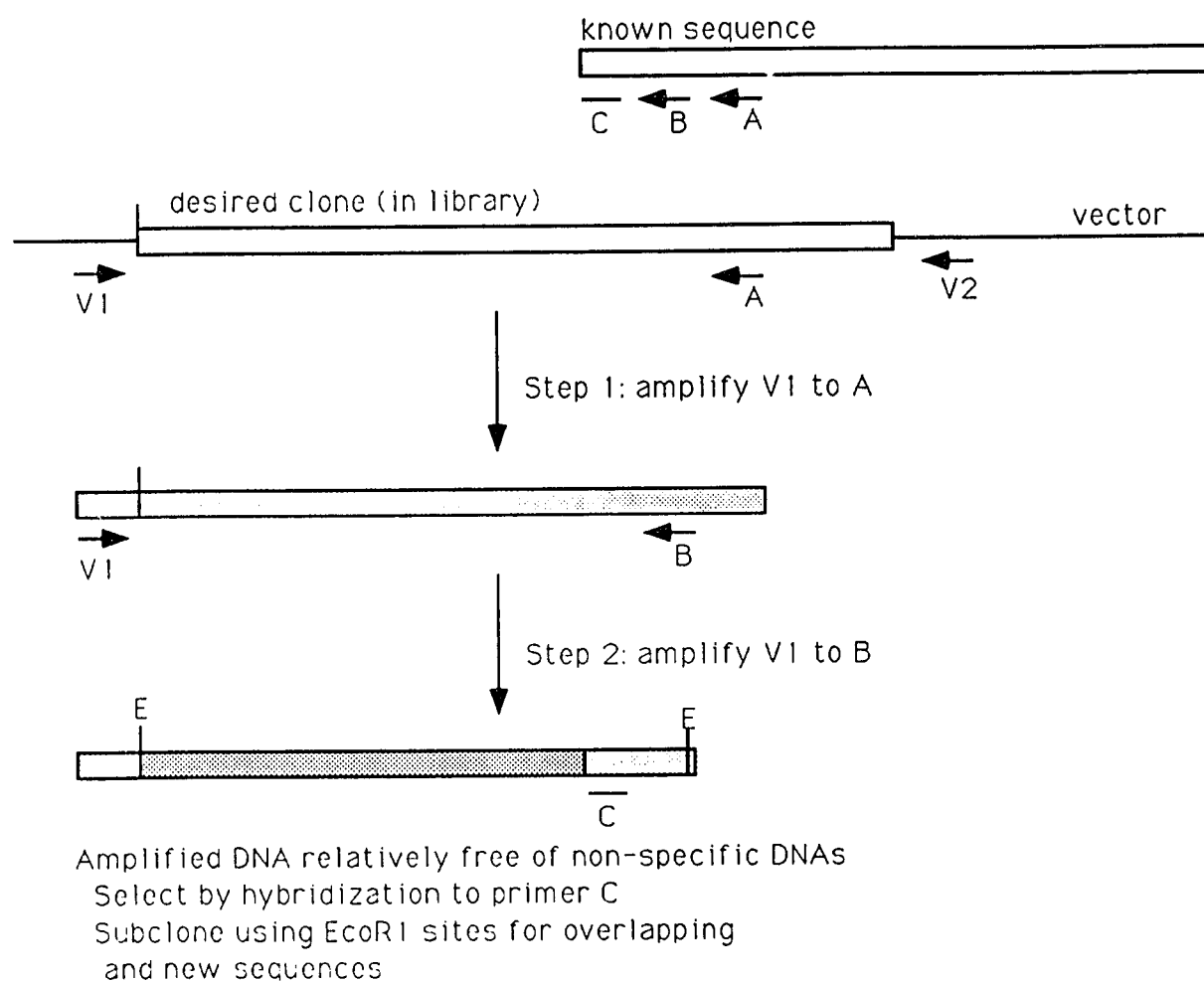
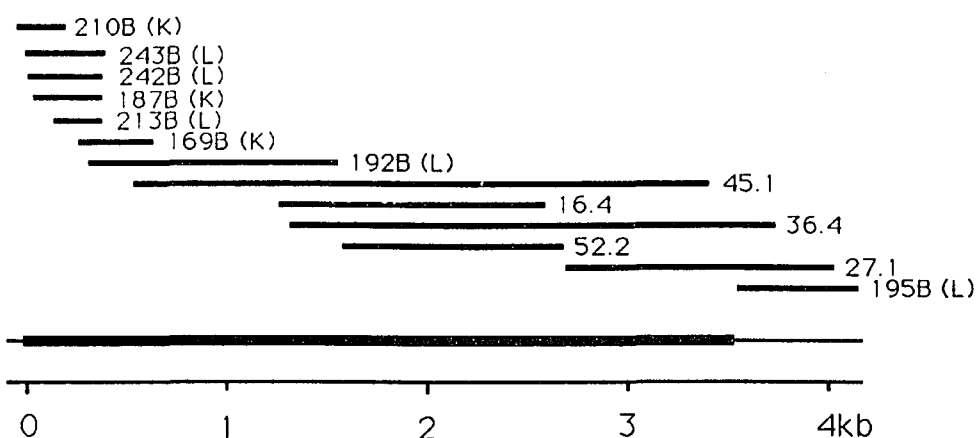
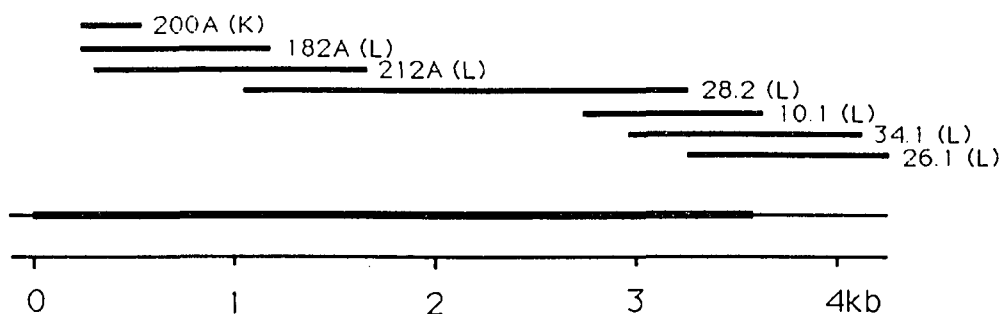


Figure 6-3. Schematic diagram of clones obtained and sequenced to determine the complete sequence of the liver types 5 and 6 adenylyl cyclases. [A] Clones for type 5 adenylyl cyclase. Clones 16.4, 27.1, 36.4, 45.1 and 52.2 were obtained by hybridization screening of a liver cDNA library. Clones 169B, 187B, and 210B were amplified by nested PCR from a rat kidney cDNA library, and clones 192B, 213B, 242B and 243B were amplified from liver first-strand cDNA to confirm previously amplified sequences. [B] Clones for type 6 adenylyl cyclase. Clones 10.1, 26.1, 28.2 and 34.1 were obtained by hybridization screening of a liver cDNA library. Clones 182A and 200A were amplified by nested PCR from a rat kidney cDNA library, and clone 212A was amplified from liver first-strand cDNA to confirm previously amplified sequences.

### Cloning Strategy for Type 5 Adenylyl Cyclase



### Cloning Strategy for Type 6 Adenylyl Cyclase



hybridization to a third adenylyl cyclase sequence oligonucleotide probe, nested within and distinct from both PCR primer sequences. Several products of considerable length were identified for both the types 5 and 6 adenylyl cyclase 5' end sequences. Products were gel purified from a second gel and subcloned using EcoR1 restriction sites amplified from the vector cloning site and built into the second-round adenylyl cyclase primers. Sequencing of several of these products has overlapped portions of the known sequences and identified additional 5'-end sequences of both adenylyl cyclase clones. Several such steps were performed for both the type 5 and 6 sequences to obtain further coding regions. Sequences derived from PCR amplified DNA bands were verified by sequencing overlapping, independent PCR products in the regions of interest. The clones which were identified for both types of adenylyl cyclase by hybridization screening and by PCR amplification are shown schematically in Figure 6-3.

#### Cloning of S49 murine lymphoma cell adenylyl cyclase: PCR amplification of cDNA

Since certain of the phosphorylation experiments described in the last chapter were carried out in the murine S49 lymphoma cell line, it was of interest to know the structure of adenylyl cyclase in these cells. To rapidly identify S49 cell adenylyl cyclase sequence, PCR amplification from S49 cell first-strand cDNA was performed using oligonucleotide primers designed to amplify several distinct adenylyl cyclases.

S49 *UNC* cell poly(A) RNA was prepared and 1 µg was reverse transcribed from a modified oligo-(dT)<sub>18</sub> primer with MMLV reverse transcriptase. The primer sequence was 5'-CATTCAGACCGATGCCACTCGAGGCTTTTTTTTTTTTTTTTTT-3', such that

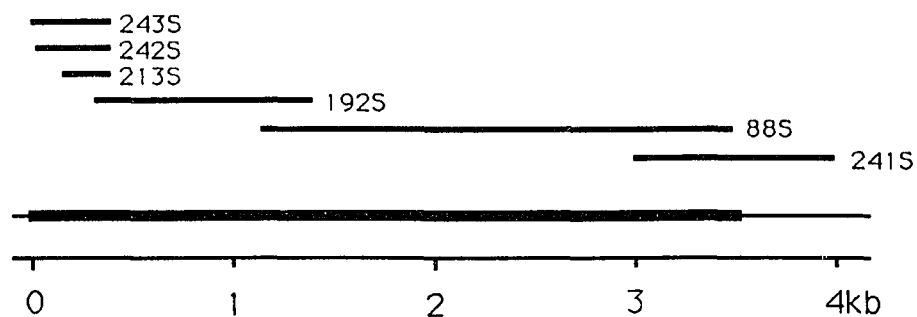
all first-strand cDNAs contain this extra 25 nucleotide sequence at their 5' ends. The central core of adenylyl cyclase, from the beginning of the first large cytoplasmic domain to very nearly the end of the coding region, was amplified by PCR from first strand cDNA using primers (88→44) which recognize domains conserved in most, but not all, cloned adenylyl cyclases. A single 2.3 kb product was obtained and subcloned using restriction sites built into the PCR primers, yielding a single class of adenylyl cyclase-related sequence which was found to be nearly identical to the rat type 5 sequence.

To amplify and clone more of this S49 cell adenylyl cyclase, S49 cell first-strand cDNA was amplified using a variety of rat type 5 adenylyl cyclase sequence primers. Most reactions were successful, and several products were subcloned for sequencing. All but the extreme ends of the sequence has been obtained in this manner.

The extreme 3'-end sequence of the S49 cell adenylyl cyclase was also obtained using a combination of the nested PCR screening method previously described, and the rapid amplification of cDNA ends (RACE) protocol of Frohman *et al* (1988). Basically, first-strand S49 cell cDNA was primed as described above with an extended oligo(dT)<sub>18</sub> primer containing an additional sequence. PCR amplification was performed using an adenylyl cyclase primer and a primer (#45) identical to the non-(dT)<sub>18</sub> portion of the cDNA primer. One µl of this reaction was used as template for an additional PCR amplification reaction using a nested adenylyl cyclase primer and the same cDNA end primer #45. Products were hybridized to an oligonucleotide probe, and the longest positive bands subcloned using an internal Xho1 site in the S49 cell adenylyl cyclase sequence and an Xho1 site in the cDNA end primer.

Figure 6-4. Schematic diagram of clones obtained and sequenced to determine the sequence of the mouse S49 cell adenylyl cyclase. All clones were obtained by PCR amplification of S49 *UNC* cell first-strand cDNA.

### Cloning Strategy for S49 Cell Type 5 Adenylyl Cyclase



The various clones which were obtained and sequenced to determine the nearly-complete nucleotide sequence of the mouse S49 lymphoma cell (type 5) adenylyl cyclase are shown schematically in Figure 6-4.

#### Sequences of the rat liver and S49 cell adenylyl cyclases

The nucleotide and deduced amino acid sequences of the two rat liver adenylyl cyclase cDNAs (types 5 and 6) and the S49 cell type 5 adenylyl cyclase partial cDNA are shown in Figures 6-5 through 6-7, respectively. Several of the clones obtained from library screening contain RNA processing artefacts (introns, improper splicing sites with loss of coding sequence). The positions of these irregularities are probably the site of intron-exon boundaries in the type 5 and 6 adenylyl cyclase genes rather than true alternative splicing, since most occur in otherwise highly conserved domains. None of these irregularities were observed in independent overlapping clones or when sequences flanking these sites were amplified by PCR.

The rat type 5 adenylyl cyclase is predicted to be 1180 amino acids in length (although no in-frame stop codon precedes the presumed initiator methionine and the coding sequence may extend even further 5' of the clones already obtained), with a calculated molecular weight of 131,978 Da. The rat type 6 adenylyl cyclase is >1098 amino acids long (no Met residues have yet been found in the sequence 5' to the first predicted transmembrane span), and has a predicted weight of >122,727 Da. The extreme 5' and 3' end sequences of the mouse (S49 cell) type 5 adenylyl cyclase were used as the sites for PCR primers to amplify internal sequences, and so these sequences are

**Figure 6-5.** Nucleotide and deduced amino acid sequence of the rat type 5 adenylyl cyclase. Amino acids are shown using the one-letter code. The first methionine is presumed to be the initiator methionine, although no upstream in-frame stop codon has been found.

|                                                                                             |      |
|---------------------------------------------------------------------------------------------|------|
| GGCGGCACCAAGAATGCCCTGCCCTGGCCCGCATCCGGTCTTGGGCGCAGCAGCATGTGTCATGGTTTAGCGGCCTCCTGGTTCCCAAAGT | 100  |
| M P L P V A R S G S G R S S M S W F S G L L V P K V                                         | 26   |
| GGATGAACGGAAACAGCCTGGGGCGAAGCAATGGACAGAAGCGCCACGCCAGCGCAGCCGAGCCCGTGGCTTCTGCGCGCCCGGCTA     | 180  |
| D E R K T A W G E R N G Q K R P R Q A T R A R G F C A P R Y                                 | 56   |
| CATGAGCTGCCTCAAGAATGTGGAGCCACCCAGCCCCACTCCTGCAGCTCGCACTCGGTGCCCTGGCAGGATGAAGCCTTCATCAGGAG   | 270  |
| M S C L L K N N V E P P S P T P A A R T R C P W Q D E A F I R R                             | 86   |
| GGCTGCCCGGGGAAGGGTGTGGAGCTGGGGCTGCGGTTCAGTGGCCTTTGGGTTTGTATGACACTGAGGTGACCAACCCGATGGGGACAGC | 360  |
| A G P G G R G V E L G L G R S V A L G F D D T E V T T P M G T A                             | 116  |
| TGAAGTGGCACCCGACACATCGCCTCGAAGCGGTCCGTCTCTGCTGGCACCGGTAGCGCAGGTGTTCCAGTCTAAGCAGTTCCTCGTCCGC | 450  |
| E V A P D T S P R S G P S C W H R L A Q V F Q S K Q F P S A                                 | 146  |
| CAAGCTGGAGCGTCTGTACACGCGGTACTTCTTCCAGATGAACCAGAGCAGCCTCACGCTGCTCATGGCGGTGCTTGTGCTCCTCATGGC  | 540  |
| K L E R L Y Q R Y F F Q M N Q S S L T L L M A V L V L L M A                                 | 176  |
| TGTACTGTTGACCTTCCACGCGCGCCTGCCCTGCCTCAGCCTGCTTATGTGGCCCTGCTGACCTGTGCCCTCCGTCCTTTTGTGGTACT   | 630  |
| V L L T F H A A P A L P Q P A Y V A L L T C A S V L F V V L                                 | 206  |
| CATGGTAGTGTGTAACCGACATAGCTTCCGCCAGGACTCCATGTGGGTAGTGAGCTATGTGGTCTTGGGCATCCTAGCAGCCGTGCAAGT  | 720  |
| M V V C N R H S F R Q D S M W V S Y V V L G I L A A A V Q T                                 | 236  |
| CGGGGGTGCCCTGGCAGCCAACCCAGCAGCCTTCAGCAGGCCTTTGGTGCCCCGTGTTCTTCTGCTACATCACCTACACACTTCTTCC    | 810  |
| G G A L A A N P R S P S A G L W C P V G F T F V Y I T Y T L L P                             | 266  |
| CATTCTGATGCGAGCGGCCGTGCTCAGTGGCCTGGGTCTTTCCACCCTGCATTGATTTTGGCCTGGCATCTCAACAATGGTGACCCCTT   | 900  |
| I R M R A A V L S G L G L S T L H L I L A W H L N N G D P F                                 | 296  |
| CCTTTGGAAGCAGCTCGGTGCTAACGTGGTGCTCTTCTGTGCACCAATGCCATCGGTGCTGTCACGCACTACCCCGCTGAAGTGCTCTCA  | 990  |
| L W K Q L G A N V V L F L C T N A I G V C T H Y P A E V S Q                                 | 326  |
| GCGCCAAGCCTTTCAGGAGACCCGTGGTTACATCCAGGCCCGGCTGCATTTGAGCATGAGAATCGACAGCAGGAACGGCTGCTGCTGTC   | 1080 |
| R Q A F Q E T R G Y I Q A R L H L Q H E N R Q Q E R L L L S                                 | 356  |
| GGTGTGCCCCAGCATGTTGCCATGAAATGAAGAGGATATCAACACAAAAAGGAAGACATGATGTTCCACAAGATTACATCCAGAA       | 1170 |
| V L P Q H V A M E M K E D I N T K K E D M M F H K I Y I Q K                                 | 386  |
| GCATGACAATGTCAGCATCTCTGTTTGGCCAGCATCGAGGGCTTACCAGCCTGGCCTCCCACTGCACTGCCAGAACCTGGTCAATGACCTT | 1260 |
| H D N V S I L F A D I E G F T C S L A S Q C A C A G L V M T L                               | 416  |
| GAATGAGCTCTTTGCCCGGTTTCGACAAGCTGGCTGCGGAGAATCACTGTCTGAGGATCAAGATCTTAGGAGACTGTTACTACTGTGTGTC | 1350 |
| N E L F A R F D K L A A E N H C L R I K I L G D C Y Y C V S                                 | 446  |
| GGGGCTGCGGAGGCCCGGGCAGACCATGCCACTGCTGTGTGGAGATGGGGGTAGACATGATCGAGGCCATCTCGTGGTGCCTGAGGT     | 1440 |
| G L P E A R A D H A H C C V E M G V D M I E A I S L V R E V                                 | 476  |
| AACGGGTGTAAATGTGAACATGCGCGTGGGCATCCACAGCGGGCGTGTAAGCTGCGGTGTCCTTGGTCTGCGGAAATGGCAGTTTGATGT  | 1530 |
| T G V N V N M R V G I H S G R V H C G V L G L R K W Q F D V                                 | 506  |
| CTGGTCCAACGATGTGACCTTGCCCAACCACATGGAGGCGGGGGCGGGCGGGCGCATCCACATCACTCGGGCCACACTGCAGTACCT     | 1620 |
| W S N D V T L A N H M E A G G R A G R I H I T R A T L Q Y L                                 | 536  |
| GAACGGGGACTATGAGGTGGAGCCAGGCCGTGGCGGTGAGCGCAACGCGTACCTCAAGGAGCAGTGCAATTGAGACCTTCCTCATACTAGG | 1710 |
| N G D Y E V E P G R G G E R N A Y L K E Q C I E T F L I L G                                 | 566  |
| AGCCAGCCAGAAACGGAAGAGGAGAAAGGCCATGCTGGTCAAGCTGCAGCGGACGCGGCCAAGCTCCATGGAAGGAGTATGCCCCCGCTG  | 1800 |
| A S Q Q K A K E E K A M L V K L G Q R T R A N S M E G L M P R W                             | 596  |
| GGTTCCTGACCGTGCTTCTCCTCCGACCAAGGACTCTAAGGCATTCCGACAGATGGGCATCGATGACTCTAGCAAGAGAACCGGGGTGC   | 1890 |
| V P D R A F S R T K D S K A F R Q M G I D D S S K E N R G A                                 | 626  |
| CCAAGATGCTCTGAACCTGAGGATGAGGTGGACGAGTTTCTGGGCCAGGCATCGATGCCCCAAGCATCGACCAGCTGCGTAAGGACCA    | 1980 |
| Q D A L N P E D E V D E F L G R A I D A R S I D Q L R K D H                                 | 656  |
| TGTGCGCGCGTTCTGCTCACCTTCCAGAGGGAGGATCTCGAGAAGAAGTATTACCGGAAAGTAGACCCTCGTTTCGGAGCCTACGTGCG   | 2070 |
| V R R F L L T F Q R E D L E K K Y S R K V D P R F G A Y V A                                 | 686  |
| CTGTGCCCTCCTGGTTTCTGCTTCATCTGTTTCATCCAGTTCCTCGTATTCCACACATCCGCGCTGATACTCGGGATTATGCCGGGAT    | 2160 |
| C A L L V F C F I C F I Q F L V F P H S A A C C I L G I Y A G I                             | 716  |
| CTTCCCTTTTGTGCTGGTCACCGTGCTCATCTGTGCTGTGCTCCTTGGGGTCTTTCTTCCCAACGCCCTCGAGCGCCTGTCCCGCAG     | 2250 |
| F L L L V V T V L I C A V C S C G S F F P N A L Q R L L S R S                               | 746  |

TATCGTCCGCTCACGGGTGCACAGCACGGCTGTTGGAGTCTTCTCGGTTCTGCTTGTGTTTCATCTCTGCCATTGCCAACATGTTACCTG 2340  
 I V R S R V H S T A V G V F S V L L V F I S A I A N M F T C 776  
 CAGTCACACCCCACTGAGGACCTGTGCGGCCCGGATGCTGAAGCTTAACACCGTCCGATGTCACCGCCTGCCACCTACGACAGCTCAATTA 2430  
 S H T P L R T C A A R M L N L T P S D V T A C H L R Q L N Y 806  
 CTCTCTGGGACTGGAAGCTCCCCTGTGTGAGGGCACCGCACCCACCTGCAGCTTCCCTGAGTACTTTGTGCGGGAGTGTGCTGTGAGTCT 2520  
 S L G L E A P L C E G T A P T C S F P E Y F V G S V L L S L 836  
 CTTGGCCAGCTCCGCTTCTCCACATCAGCAGCATTGGCAAGCTAGTTATGACCTTTGTCTTGGGGTTTCATCTACTTGTCTTCTGCTTTT 2610  
 L A S S V F L H I S S I G K L V M T F V L G F I Y L L L L L 866  
 GCTGGTCCCCAGCCACCATCTTTGACAACTATGATCTACTGCTTAGCGTCCATGGCTTGGCTTCCCTCCAATGAGACCTTCGATGGGCT 2700  
 L G P P A T I F D N Y D L L L S V H G L A S S N E T F D G L 896  
 GGACTGCCAGCCGTAGGGAGGGTAGCGCTCAAATACATGACCCCTGTGATTCTCTCGTGTTCGCCCTGGCACTGTATCTACACGCACA 2790  
 D C P A V G R V A L K Y M T P V I L L V F A L A L Y L H A Q 926  
 ACAGGTGGAATCTACCGCCCGCTGGACTTCTGTGGAAGTGCAGGCCACAGGGGAGAAGGAGGAGATGGAGGAGTTGCAGGCCCTACAA 2880  
 Q V E S T A R L D F L W K L Q A T G E K E E M E E L Q A Y N 956  
 CCGGCGGCTGTGCTAATACATCCTTCCCAAGGACGTGGCTGCCCACTTCTGTGCGCCGGGAGCGCCGCAACGACGAGCTGTACTACCAATC 2970  
 R R L L H N I L P K D V A A H F L A R E R R N D E L Y Y Q S 986  
 CTGCGAGTGCCTGGCTGTCTATGTTTGCCTCCATCGCCAACTTCTCTGAGTTCATGTGGAAGTGGAGGCGAACAATGAGGCGTGGAGTG 3060  
 C E C V A V M F A S I A N F S E F Y V E L E A N N E G V E C 1016  
 CCTGCGACTGTCTAATGAGATCATCGCGGACTTTGATGAGATCATCAGTGAGGAGAGGTTCCGCGAGCTGGAGAAGATCAAGACCATCGG 3150  
 L R L L N E I I A D F D E I I S E E R F R Q L E K I K T I G 1046  
 TAGCACTTACATGGCCCGCTCCGGGCTAAATGCCAGCACCTATGACAGGTGCGCCGCTCGCACATCACCGCCCTGGCAGACTACGCCAT 3240  
 S T Y M A A S G L N A S T Y D Q V G R S H I T A L A D Y A M 1076  
 GCGGCTTATGGAGCAAATGAAACACATCAACGAACACTCTTTCAACAACCTCCAGATGAAGATCGGGTTGAACATGGGTCCGGTTGTAGC 3330  
 R L M E Q M K H I N E H S F N N F Q M K I G L N M G P V V A 1106  
 AGGTGTCATTGGGGCCCGAAGCCACAGTATGACATCTGGGGAACACGGTGAATGTTTCCAGCCGTATGGACAGCACAGGAGTTCTGA 3420  
 G V I G A R K P Q Y D I W G N T V N V S S R M D S T G V P D 1136  
 CCGAATACAGGTGACCACGGATCTCTACAGGTCTAGCTGCCAAGGGCTACCAACTGGAGTGTGAGGGGTGGTCAAGGTGAAGGAAA 3510  
 R I Q V T T D L Y Q V L A A K G Y Q L E C R G V V K V K G K 1166  
 GGGGAGATGACCACCTACTTCTCAATGGGGGCCCCAGCAGTTAGCAGAGCGCAGCAGTGAAATTCAACCAAGGGACCAAGGTGGGC 3600  
 G E M T T Y F L N G G P S S \* 1180  
 ACTGAGTGGACTTTTCTGCTCACTGGATGGAGCTGTGGCAGGGGGCTCTGAGCCTTCAGGTCTTGCTGACAGCAAAAGGGAACACCCAG 3690  
 CAGGCTGTGCTTGGACCATATCCGTCTGCCCTCAGAGCAGCAAAGGAAGGACGCCGAGAGGATTATTATCCAAGTGACTTTCTTAATCA 3780  
 GAGTAAGGCTGTTTGCTTTTTTTTCCCCCTTTGGACGCAGCTGAGAGCAGGCCTCCTGTTTGAGAGTAAATGGCAACTTACTCTGC 3870  
 CTACTGTTTCCCTGTCTGGGCGACAGGCTCAGGGCTGGGACCTTTCTTCCCTATTTTTCTGGGACTATTTTGTACAAGAGTGGGGCA 3960  
 GGCGATGAGGAATGCTTGCCCTTCACTGGCCTGTGTCAGCAGCATTGTCTTGGGCACTCCAGTACCAGCCAAGTCTCCTTCTAGCACAA 4050  
 CAAGGCAGAGGAAGGAGGTGCTGTGGGACCTAGCTCTGACCAGATTTTCAGGGAATGTTTCCATTTGCCAAATCCTAGTCC 4131

Figure 6-6. Nucleotide and deduced amino acid sequence of the rat type 6 adenylyl cyclase. Amino acids are shown using the one-letter code. No candidate initiator methionine has yet been found in currently cloned 5' fragments.

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GTCGAGGAAAAGGCCGAGCGGCCGAGGAGCTGGAGCCCGGAAGTGGCACCGTCGAGGATGGAGACGGGTCCGAGGATGGAGGCAGTTCCG 90
R G K G R A A E E L E P G T G T V E D G D G S E D G G S S V 30
TGGCGTCAGGCTCTGGGACCGGCACGGTGTGTCGTTGGGGCGCTGCTGCCTGGCCTTGCTGCAGATATTCGGCTCTAAGAAGTTCCCGT 180
A S G S G T G T V L S L G A C C L A L L Q I F R S K K F P S 60
CGGACAAACTGGAGCGTCTGTACCAGCGCTATTTCTTCCGCCCTGAACAGAGCAGCCTCACCATGCTCATGGCCGTGCTGGTGTCTGCTGT 270
D K L E R L Y Q R Y F L R L N Q S S L T M L M A V L V L V C 90
GCCTGGTTCATGCTCGCTTTCCACGCGGCACGGCCCCCGCTCCAGGTAGTCTACCTGGCCGTGTTGGCAGCTGCTGTGGGCGTGATCCTTA 360
L V M L A F H A A R P P L Q V V Y L A V L A A A V G V I L I 120
TCATGGCTGTGCTCTGCAACCGTGCAGCCTTCCACCAGGACCACATGGGCCTGGCCTGCTATGCGCTCATTGCAGTGGTGTGGCCGTCC 450
M A V L C N R A A F H Q D H M G L A C Y A L I A V V L A V Q 150
AGGTAGTGGGCTGTGTGCTGCCACAGCCAGCAGCGCCTCCGAGGGCATCTGGTGGACCGTGTCTTCATCTATACCATCTACACCGTGC 540
V V G L L L P Q P R S A S E G I W W T V F F I Y T I Y T L L 180
TGCCTGTGCGCATGAGGGCTGCGGTGCTCAGCGGGGTGCTTCTGTGGGCTCTCCACTTGGCCATCTCTCTGCACACCAACGCCAGGACC 630
P V R M R A A V L S G V L L S A L H L A I S L H T N A Q D G 210
AGTTCTGCTGAAACAGCTTGTCTCCAACGTCCTCATCTTCTCTTGCACCAACATCGTGGGTGTGTGCACTCACTACCCAGCCGAGGTCT 720
F L L K Q L V S N V L I F S C T N I V G V C T H Y P A E V S 240
CCCAGAGACAGGCCTTCCAAGAGACCCGGGAGTGCATCCAAGCTCGGCTTCACTACAGCGGGAGAACCAGCAACAGGAGCGTCTCTCTGC 810
Q R Q A F Q E T R E C I Q A R L H S Q R E N Q Q Q E R L L L 270
TGCTGTCTCTTCCCGTCATGTTGCCATGGAGATGAAAGCAGACATCAACGCCAAACAGGAGGATATGATGTTCCACAAGATTACATCC 900
S V L P R H V A M E M K A D I N A K Q E D M M F H K I Y I Q 300
AGAAACATGACAATGTGAGCATCTGTGTTGCTGACATCGAAGGCTTCACTAGCCTGGCATCCCAGTGTAAGTCCCAAGAACTGGTCATGA 990
K H D N V S I L F A D I E G F T S L A S Q C T A Q E L V M T 330
CCCTCAACGAGCTCTTCGCCCCGCTTTGACAAGTTGGCTGCGGAGAATCACTGCTTACGGATTAAAGATCCTCGGGGATTGTTACTACTGTG 1080
L N E L F A R F D K L A A E N H C L R I K I L G D C Y C V 360
TCTCGGGCTGCCTGAAGCCAGAGCTGACACGCCCCACTGCTGCTAGAGATGGGAATGGACATGATCGAGGCCATCTCGTGGTCCGGG 1170
S G L P E A R A D H A H C C V E M G M D M I E A I S S V R E 390
AGGTGACAGGGGTGAACGTGAACATGCGGTGTGGGAATTCACAGCGGGAGAGTACATGCGGTGTCTTGGCCTCAGAAAGTGGCAATTCG 1260
V T G V N V N M R V G I H S G R V H C G V L G L R K W Q F D 420
ACGTGTGCTTAAACGATGTACACTGGCCAAACCATGGAAGCTGGCGGAAGGCAGGCCGATCCACATCACCAAGGCCACACTCAACT 1350
V W S N D V T L A N H M E A G G K A G R I H I T K A T L N Y 450
ACCTGAACGCGGACTATGAGGTGGAGCCAGGCTGTGGTGGTGGAGCGCAATGCTTACCTCAAGGAGCAGCATCGAGACCTTCCTCATCC 1440
L N G D Y E V E P G C G E R N A Y L K E H S I E T F L I L 480
TGGCTGTATCCAGAAAGCGGAAAGAAGAGAGGCCATGATCGCCAAGATGAACCGCCAGAGAACCAACTCCATTGGACACAATCCGCCCTC 1530
R C T Q K R K E E K A M I A K M N R Q R T N S I G H N P P H 510
ACTGGGGAGCTGAGCGCCCTTCTACAACCACTTGGGTGGCAACCAGGTGTCCAAGGAAATGAAGAGGATGGGCTTTGAGGACCCCAAG 1620
W G A E R P F Y N H L G G N Q V S K E M K R M G F E D P K D 540
ACAAGAATGCCAGGAAAGTGCCAACCTGAGGATGAAGTGGACGAGTTTCTGGGTGAGCCATCGATGCCAGGAGTATTGACAGACTGC 1710
K N A Q E S A N P E D E V D E F L G R A I D A R S I D R L R 570
GATCCGAACACGTCGAAAGTTCTCTTGACCTTTAGGGAGCCGACTTAGAGAAGAAGTACTCCAAGCAGGTGGATGACCGATTGGTG 1800
S E H V R K F L L T F R E P D L E K K Y S K Q V D D R F G A 600
CCTATGTGGCCTGTGCCTCGCTTGTTCCTCTTCATCTGCTTTGTCCAGATCACCATTGTGCCCCACTCCCTGTTTCATGCTGAGCTTCT 1890
Y V A C A S L V F L F I C F E R V Q I T I V P H S L F M L S F Y 630
ACCTGTGCTGTTTCTGTACTGGCCTTGGTGGTGTATATCTGTGATCTACGCCTGTGTGAAGCTCTTCCCTACTCCCCGTCAGACAC 1980
L S C F L L L A L V V F I S V I Y A C V K L F P T P L Q T L 660
TCTCCAGGAAGATAGTGGATCCAAGAAGAACAGCACCCTGGTGGGGGTGTTACCATCACCCTGGTGTCTCTCGGCTTTTGTCAACA 2070
S R K I V R S K K N S T L V G V F T I T L V F L S A F V N M 690
TGTTATGTGCAACTCTAAGAACCTGGTGGGTGGCTGGCAGAGGAGCACAACATCACGGTGAACAGGTGAACGCATGTCTATGTATGG 2160
F M C N S K N L V G C L A E E H N I T V N Q V N A C H V M E 720
AGTCGGCCTTCAACTACAGCTGGGCGACGAGCAGGGCTTCTGTGGCAGCCCCAGTCCAAGTGAACCTTCCAGAGTACTTACCTACA 2250
S A F N Y S L G D E Q G F C G S P Q S N C N F P E Y F T Y S 750

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GCGTGCTGCTCAGCCTGCTGGCCTGCTCCGTGTTCTGCGAGATCAGCTGCATCGGGAAGCTGGTGCTCATGCTGGCCATCGAGCTCATCT 2340  
 V L L S L L A C S V F L Q I S C I G K L V L M L A I E L I Y 780  
 ACGTGCTCATCGTAGAGGTGCCCGGTGTCACACTCTTTGACAACGCTGACCTTCTGGTCACCGCCAATGCCATAGACTTCAGCAACAACG 2430  
 V L I V E V P G V T L F D N A D L L V T A N A I D F S N N G 810  
 GGACCTCCCAGTGCCCTGAGCATGCGACCAAGGTGGCGCTGAAGGTGGTGACGCCCATCATCATCTCTGTCTCTGTGCTGGCTCTGTATC 2520  
 T S Q C P E H A T K V A L K V V T P I I I S V F V L A L Y L 840  
 TGCATGCTCAGCAGGTGGAATCCACTGCTCGCCTTGACTTCTCTGGAAGTGCAGGCCACAGAAGAGAAGGAGGAGATGGAGGAGCTGC 2610  
 H A Q Q V E S T A R L D F L W K L Q A T E E K E E M E E L Q 870  
 AGGCCTACAACCGCGCTTGTGTCACAACATTCTGCCCCAAGGACGTGGCTGCCCACTTCTGGCCCGGAGCGCGCAATGATGAACGTGT 2700  
 A Y N R R L L H N I L P K D V A A H F L A R E R R N D E L Y 900  
 ACTACCAATCCTGCGAGTGCGTGGCTGTCATGTTTGCCTCCATCGCCAATCTTCCGAATTCTACGTGGAGCTAGAGGCCAACAATGAGG 2790  
 Y Q S C E C V A V M F A S I A N F S E F Y V E L E A N N E G 930  
 GCGTTGAATGCCTACGGCTGCTCAATGAGATCATCGCAGACTTTGATGAGATCATCAGTGAGGATCGGTTTCAGGCAGCTGGAAAAGATCA 2880  
 V E C L R L L N E I I A D F D E I I S E D R F R Q L E K I K 960  
 AGACCATAGGTAGCACCTACATGGCTGCCCTTGGCCTCAACGACTCCACCTATGACAAGGCAGGCAAGACCCACATCAAGGCTCTTGAG 2970  
 T I G S T Y M A A S G L N D S T Y D K A G K T H I K A L A D 990  
 ACTTCGCCATGAAGCTGATGGACCAATGAAGTACATCAATGAGCACTCCTTCAACAACCTCCAGATGAAGATCGGGCTTAACATTGGAC 3060  
 F A M K L M D Q M K Y I N E H S F N N F Q M K I G L N I G P 1020  
 CTGTAGTGGCTGGGGTCATTGGGGCTCGCAAGCCTCAGTATGACATCTGGGGCAATACAGTAAATGTGGCCAGCCGATGGACAGCACTG 3150  
 V V A G V I G A R K P Q Y D I W G N T V N V A S R M D S T G 1050  
 GGGTGCCTGACCGCATCCAGGTTACTACAGATATGTACCAGGTGCTGGCCGCCAACACATACCAGCTGGAGTGGCGGGGTGTGGTCAAGG 3240  
 V P D R I Q V T T D M Y Q V L A A N T Y Q L E C R G V V K V 1080  
 TCAAGGGCAAGGGTGAATGATGACCTACTTCTCAATGGAGGGCTCCCTCAGTTAGCAGATACCAGCAGCATCCTGGCCTCCAGAGG 3330  
 K G K G E M M T Y F L N G G P P L S \* 1098  
 ATTGAACAGCTTCTCTGTGCACCATGGGCAGATAGGGCCTGTGCCAGGCTCACAGCTGTGCTGGTAAAATTTCTACTTGGACTCAGAAG 3420  
 CAACTTCTGCCTTTGCTGGTGGGCATGCATTTTGGCCTAGGCCAGGGTGCCAGCGTCTGCGAGCACCGAGCTGACCAAGATGTTGCCC 3510  
 TTGGCAGAAGACTGTAGACTCGAGCTGACCCCTTGAGGTTCTGACAAGTGCTGCTACTGCACGGTGGATGTGCTACCGGAGCACAGCCTGG 3600  
 CACAGAGTGGCTGGCCTGAGGGGAGGCCTTGGTTATGAAGGGGAGGTAGGCAAGCTTGAAGCTGGCCTGGGGGATGTCCATGGACCTCAT 3690  
 GGGTCTGCTTTCCACTGTGGAGAGGCTATGCCCCCTCACAACGTTTGGGGGAGGAACTGGGAGAGATGCGGCCCTGTGCCATTCTCTCC 3780  
 TCACACCTCCATGCACACAGACAATGCCCTGTACGGGAAACAGGACTGTTGATAAGGGGGAGGCAAGAGGACACCAGGCAAGGAGCAGTG 3870  
 GCTCTGAGCAAAAGAAAATATTTATTAATAAAACAAAAGTTTCTCTGCCCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3956

Figure 6-7. Nucleotide and deduced amino acid sequence of the mouse (UNC S49 lymphoma cell) type 5 adenylyl cyclase. Amino acids are shown using the one-letter code. The positions of oligonucleotide PCR primers used to amplify the extreme end sequences are indicated with '#' and the corresponding amino acids in the rat type 5 adenylyl cyclase sequence are shown in *italic*. The first Met residue shown corresponds to Met<sup>57</sup> of the rat type 5 adenylyl cyclase (Figure 6-5). Additional sequences from both the 5' and 3' ends have been amplified by PCR from S49 cell first-strand cDNA, but have not yet been sequenced.

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#####CGGACGGACCCAGCCCCACTCCTGCAGCTCGCACTCGGTGCCCTGGCAGGATGAAGCCTTCATCAGGAGG 90
M S C L K N A D G P S P T P A A R T R C P W Q D E A F I R R 23

GCGGGCCCGGCCAGGGGTGTGGAGCTGGGGCTGCGGTCACTGGGCTTGGGGTTTGACGACACTGAGGTGACCACACCGATGGGCACAGCT 180
A G P A R G V E L G L R S V A L G F D D T E V T T P M G T A 53

GAAGTGGCACCAGGATACATCGCCTCGGAGCGGTCCGTCTGCTGGCAGCGGCTTGTGCAGGTGTTCCAGTCTAAGCAGTTCGCTCTGCC 270
E V A P D T S P R S G P S C W H R L V Q V F Q S K Q F R S A 83

AAGCTGGAGCGGGTGTACAGCGGTACTTCTCCAGATGAACCAGAGCAGCCTCACGCTGCTCATGGCGGTGCTGGTGTCTCATGGCT 360
K L E R L Y Q R Y F F Q M N Q S S L T L L M A V L V L L M A 113

GTACTGTTGACTTTCACGCTGCGCCTGCCAGCCTCAGCCTGCTTACGTGGCCCTGCTGACCTGTGCCTCTGCTCTTTTGTGGTACTC 450
V L L T F H A A P A Q P Q P A Y V A L L T C A S V L F V V L 143

ATGGTGGTGTGTAACCGACACAGCTTCCGCCAGGACTCCATGTGGTGGTGAGCTATGTGGTCTGGGCATCCTAGCAGCCGTGCAAGTC 540
M V V C N R H S F R Q D S M W V V S Y V V L G I L A A V Q V 173

GGGGGTGCCCTGGCAGCAATCCACACAGCCCTCGGCGGGCCTTTGGTGGCCCGTCTTCTGCTCTACATCACCTACACTCTTCTTCCC 630
G G A L A A N P H S P S A G L W C P V F F V Y I T Y T L L P 203

ATTGCGATGCGAGCCGAGTACTCAGCGCCCTGGGCCCTCTCTACTCTGCATTGATTTTGGCCTGGCAGCTCAACAGCAGCGACCCCTTC 720
I R M R A A V L S G L G L S T L H L I L A W Q L N S S D P F 233

CTTTGAAGCAGCTCGGTGCTAACGTGGTGTCTTCTCTGCAACCAATGCCATCGGTGTCTGCACACACTACCTGTGAAGTGTCTCAG 810
L W K Q L G A N V V L F L C T N A I G V C T H Y P A E V S Q 263

CGCCAAGCCTTTAGGAGACCCGAGGTACATCCAGGCGGGCTGCACCTGCAGCATGAGAACCGTCAGCAGGAACGGTGTGTATCG 900
R Q A F Q E T R G Y I Q A R L H L Q H E N R Q Q E R L L L S 293

GTGTTGCCCCAGCAGCTTGCCATGGAGATGAAAGAAGACATCAACACAAAAAGAGGACATGATGTTCCATAAGATCTACATCCAGAAG 990
V L P Q H V A M E M K E D I N T K K E D M M F H K I Y I Q K 323

CATGATAATGTACAGCATCTGTTTTCGGACATTGAGGGCTTACCAGCCTGGCCTCCAGTGCAGTGCACAGGAAGTGGTCATGACCTTG 1080
H D N V S I L F A D I E G F T S L A S Q C T A Q E L V M T L 353

AATGAGCTCTTTGCCCGGTTTGACAAGCTGGCTGCGGAGAACTCACTGTCTGAGGATCAAGATCTTAGGAGACTGTTACTACTGCGTGTCA 1170
N E L F A F D K L A A E N H C L R I K I L G D C Y Y C V S 383

GGGCTGCCCGAGGCCCGGCAGATCACGCCCACTGCTGTGTGGAGATGGGCGTAGACATGATCGAAGCCATCTCGCTGGTGGTGGAGTA 1260
G L P E A R A D H A H C C V E M G V D M I E A I S L V R E V 413

ACAGGTGTGAACGTGAACATGCGTGTGGGCATCCACAGCGGACGTGTGCATTGCGGCGTCTTGGCCTACGGAATGGCAGTTTGATGTC 1350
T G V N V N M R V G I H S G R V H C G V L G L R K W Q F D V 443

TGGTCAAACGATGTGACCTGGCTAACCATGAGAGCGGGGGGGGGCGGCCGCATCCACATCACTCGGGCTACACTGCAGTACTTG 1440
W S N D V T L A N H M E A G G R A G R I H I T R A T L Q Y L 473

AACGGGACTATGAGGTGGAGCCAGGCGGTGGTGGTGAACGCAATGCGTACCTCAAGGAGCAGTGCATTGAGACCTTCTCATACTTGGC 1530
N G D Y E V E P G R G G E R N A Y L K E Q C I E T F L I L G 503

GCCAGCAAAAACGGAAGAGGAGAAAGCCATGCTGGCCAAGCTTCAGCGGACACGGGCCAACTCCATGGAAGGACTGATGCCCCGCTGG 1620
A S Q K R K E E K A M L A K L Q R T R A N S M E G L M P R W 533

GTTCTGACCGTGCCTTCTCCCGACCAAGGACTCTAAGGCATTCCGCCAGATGGGCATTGATGATTCTAGCAAGACAACGGGGTGCC 1710
V P D R A F S R T K D S K A F R Q M G I D D S S K D N R G A 563

CAAGATGCTCTGAACCTGAAGATGAGGTGGATGAGTTCCTGGGCGGAGCCATCGATGCCGAAGCATCGACCAACTGCGTAAGGACCAT 1800
Q D A L N P E D E V D E F L G R A I D A R S I D Q L R K D H 593

GTGCGCGGTTCTGCTCACCTTCCAGAGAGAGGATCTTGAGAAGAAGTATTACGGAAGTAGATCCTCGCTTCGGAGCCTACGTCGCC 1890
V R R F L L T F Q R E D L E K K Y S R K V D P R F G A Y V A 623

TGTGCCCTCCTGGTTTTTGTCTCATCTGTTTATCCAGCTCCTTGTGTTCCCATCTCCACCTGATACTCGGGATTTATGCCGCTATC 1980
C A L L V F C F I C F I Q L L V F P Y S T L I L G I Y A A I 653
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TTCCTGCTGTTGCTGGTCACTGTGCTGATCTGTGCCGTGTGCTCCTGCGGTTCTTTCTTCCCAAGGCCCTGCAACGCCCTGTCCTCCGCAAT 2070
F L L L L V T V L I C A V C S C G S F F P K A L Q R L S R N 683

ATTGTCCGCTCACGGGGGCACAGCACCGCGGTTGGAATCTTCTCGGTTCTGCTTGTTCATCTCTGCCATCGCCAACATGTTTACCTGT 2160
I V R S R G H S T A V G I F S V L L V F I S A I A N M F T C 713

AATCACACCCCAATAAGGACCTGCGCGGCCCGGATGCTGAACCTTAACACCAGCGGATGTACCGCCTGCCACCTACAACAGCTCAATTAC 2250
N H T P I R T C A A R M L N L T P A D V T A C H L Q Q L N Y 743

TCTCTGGGACTGGATGCTCCCTGTGTGAGGGCACCGCACCCACCTGCAGCTTCCCTGAGTACTTCGTGCGGAACGTGCTGCTGAGTCTT 2340
S L G L D A P L C E G T A P T C S F P E Y F V G N V L L S L 773

CTAGCCAGCTCTGTCTTCTACACATCAGCAGCATCGGCAAGCTGGCCATGACCTTCATCTTGGGGTTCACCTACTTGGTGCTGCTTTTG 2430
L A S S V F L H I S S I G K L A M T F I L G F T Y L V L L L 803

CTGGGTCCCCCGCCGCCATCTTTGACAACATATGATCTACTGCTTGGCGTCCATGGCTTGGCTTCCCTCCAATGAGACCTTTGATGGGCAG 2520
L G P P A A I F D N Y D L L L G V H G L A S S N E T F D G Q 833

GACTGCCAGCTGTGGGGAGGGTAGCGCTCAAATATATGACCCCGTGATTCTGCTGGTGTTCCTGCCCTGGCACTGTATCTGCATGCACAA 2610
D C P A V G R V A L K Y M T P V I L L V F A L A L Y L H A Q 863

CAGGTGGAATCGACTGCCCGCCTGGACTTCTGTGGAAGTTACAGGCAACAGGGGAGAAGGAGAGATGGAGGAGCTACAGGCATACAAC 2700
Q V E S T A R L D F L W K L Q A T G E K E E M E E L Q A Y N 893

CGGAGGTTGCTGCATAACATTCTTCCCAAGGACGTGGCCGCCCACTTCTGGCCCGGAACGCCGCAACGATGAGCTGTACTACAGTCG 2790
R R L L H N I L P K D V A A H F L A R E R R N D E L Y Y Q S 923

TGTGAATGTGTGGCTGTCTGTTTGCCTCCATCGCCAATTTCTCGGAGTTCTACATGGAGCTCGAGGCAAAACAACGAGGGCGTGGAGTGC 2880
C E C V A V M F A S I A N F S E F Y M E L E A N N E G V E C 953

CTGCGGCTGCTCAATGAGATCATCGCAGACTTTGACGAGATCATCAGTGAGGAGAGATTCCGGCAGTTGGAGAAGATCAAGACCATCGGT 2970
L R L L N E I I A D F D E I I S E E R F R Q L E K I K T I G 983

AGCACCTACATGGCCGCCCTCTGGGCTAAATGCCAGCAGCTATGACCAGGTGCGGCCGCTCACACATCACGGCGCTGGCTGACTATGCCATG 3060
S T Y M A A S G L N A S T Y D Q V G R S H I T A L A D Y A M 1013

CGGCTCATGGAGCAGATGAAACACATCAATGAACACTCTTTCAACAATTTCCAGATGAAGATCGGGTTGAACATGAGTCCGGTTGTAGCA 3150
R L M E Q M K H I N E H S F N N F Q M K I G L N M S P V V A 1043

GGCGTCATTGGGGCCCGAAAGCCACAGTATGACATCTGGGGAATACCGTGAATGTTTCCAGTCGTATGGACAGCACTGGAGTTCCTGAC 3240
G V I G A R K P Q Y D I W G N T V N V S S R M D S T G V P D 1073

CGAATACAGGTGACTACGGACCTATACAGGTTCTAGCTGCCAAGGGCTACCAGCTGGAGTGTGCGAGGGGTGGTCAAG##### 3330
R I Q V T T D L Y Q V L A A K G Y Q L E C R G V V K V K G K 1099

##### 3339
G E M (11 more amino acids to end of rat type 5 adenylyl cyclase clone) 1099

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presumably similar to those of the rat type 5 sequence. The stop codons of the rat type 5 and 6 sequences are a single codon apart.

Secondary structure predictions on the two rat (and mouse S49 cell) sequences indicate the potential for two clusters of six transmembrane spans (overlined in alignment: Figure 6-8) separating two large domains predicted to be cytoplasmic (see Figure 1). Two N-linked glycosylation sites are predicted between membrane spans 9 and 10 in all three adenylyl cyclases, and one additional site is found between spans 11 and 12.

#### Comparison of adenylyl cyclase sequences: evidence for distinct sub-families

All the currently published mammalian adenylyl cyclase sequences are aligned in Figure 6-8. Localization of residues conserved among all adenylyl cyclase sequences indicates that most amino acid conservation is concentrated in the two large cytoplasmic domains, while the transmembrane spans vary greatly in their degree of conservation but in general do not appear to contain highly conserved sequences. Portions of these cytoplasmic domains are very highly conserved, with several clusters of near total conservation (indicated by '\*' in Figure 6-8). These regions of conservation among adenylyl cyclases are related to each other (quasi-duplicated) and are also clearly related to a region conserved among the several cloned guanylyl cyclases (Krupinski *et al*, 1989; Koesling *et al*, 1991). Thus the functional residues of the adenylyl cyclases are likely to be found in these cytoplasmic domains. The membrane spans appear unlikely to be directly involved in ATP binding and catalysis, or in binding  $G_s$ . Initial speculation that adenylyl cyclases may also act as a transmembrane pump or channel, based on topological

[illegible]



similarities to transporters, now appears less likely since all adenylyl cyclases have quite distinct membrane spanning regions (unless each member of the family transports distinct molecules). Interestingly, a recent report has linked a  $K^+$  channel and a hyperpolarization-stimulated adenylyl cyclase activity in a single protein purified from *Paramecium* (Schultz *et al*, 1992). Although the sequences in the first and second conserved cytoplasmic domains are clearly related to each other (quasi-duplicated), their differences are also conserved among members of the adenylyl cyclase family. This implies that while the two halves of the protein may have resulted from a duplication of a single catalytic sequence and the various adenylyl cyclase genes probably arose by gene duplication, gene conversion between the two halves of each sequence is not very common or results in loss of activity.

Pairwise matrix comparison creates a graphical representation of the similarity relationships between two sequences. Matrix comparison of the deduced amino acid sequence of the rat type 5 enzyme with the bovine brain type 1 sequence is shown in Figure 6-9. These two sequences share 52% similarity overall. Matrix comparison of the sequences of the deduced amino acid sequences of the rat liver type 5 and type 6 adenylyl cyclases makes it apparent that these two sequences are more highly related to each other than are the type 1 and type 5 sequences (Figure 6-10). The rat type 5 adenylyl cyclase has 66% overall identity and 75% similarity with the rat type 6 adenylyl cyclase. Highest similarity between these two sequences is found in the two large cytoplasmic domains, although extensive similarity is also maintained throughout portions of the transmembrane regions. The S49 cell adenylyl cyclase partial sequence shares 96% identity and 98%

Figure 6-9. Matrix comparison of rat type 5 and bovine type 1 adenylyl cyclase deduced amino acid sequences. The deduced amino acid sequences of the rat type 5 and bovine type 1 (Krupinski *et al*, 1989) adenylyl cyclases were compared using the MacVector program. Using a window of 8 amino acids, the first sequence is compared to the second. Wherever in the sequences both are greater than 60% similar over the 8 amino acid window, a dot is placed in a matrix corresponding to this position in both sequences. Thus, completely identical sequences will yield an unbroken diagonal line. The two diagonals shown represent to occurrence of conserved sequences in the two forms centered in the two large cytoplasmic domains of the enzyme.

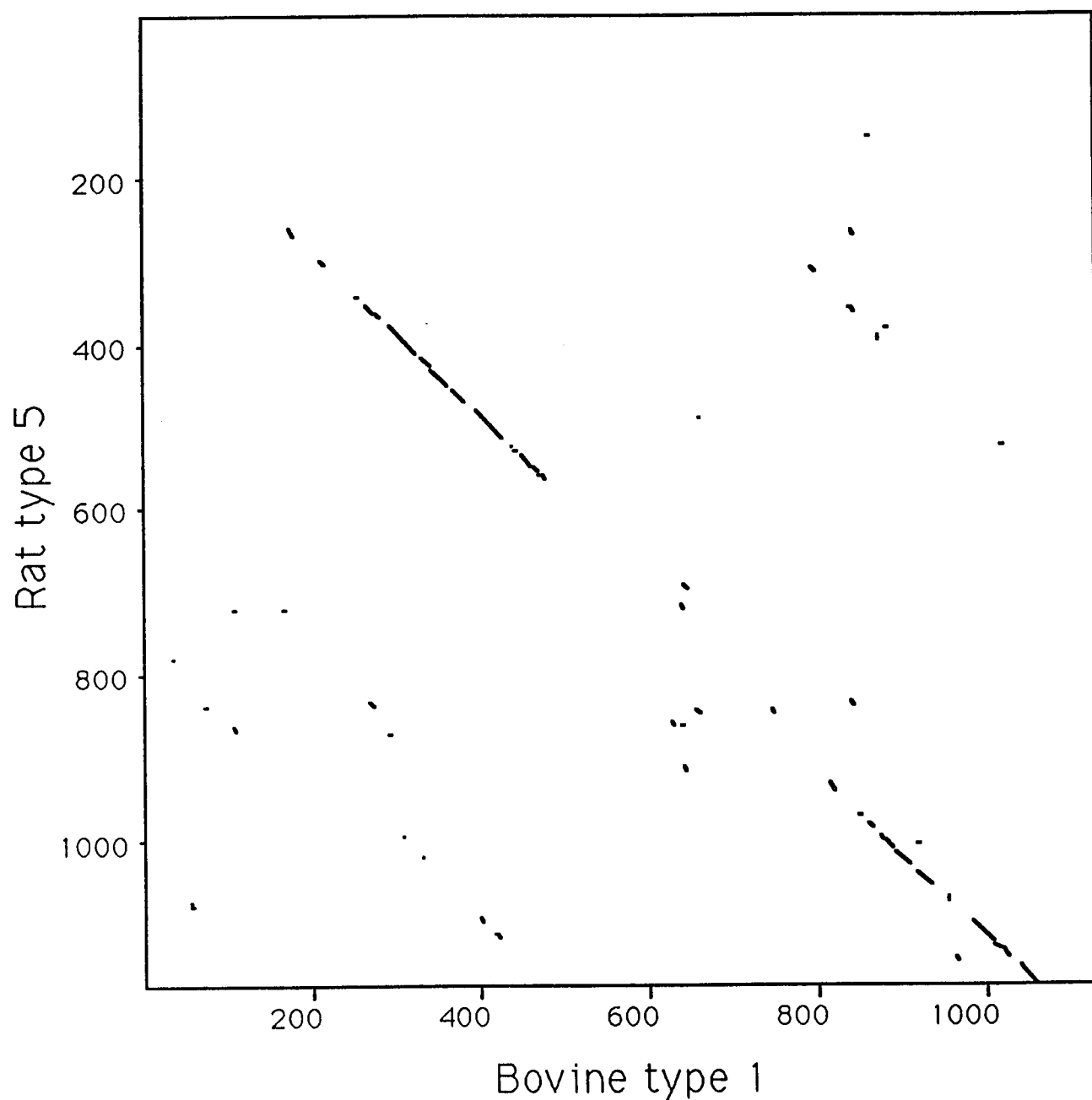


Figure 6-10. Matrix comparison of the rat type 5 and 6 adenylyl cyclase deduced amino acid sequences. Comparison conditions are as described for Figure 6-9.

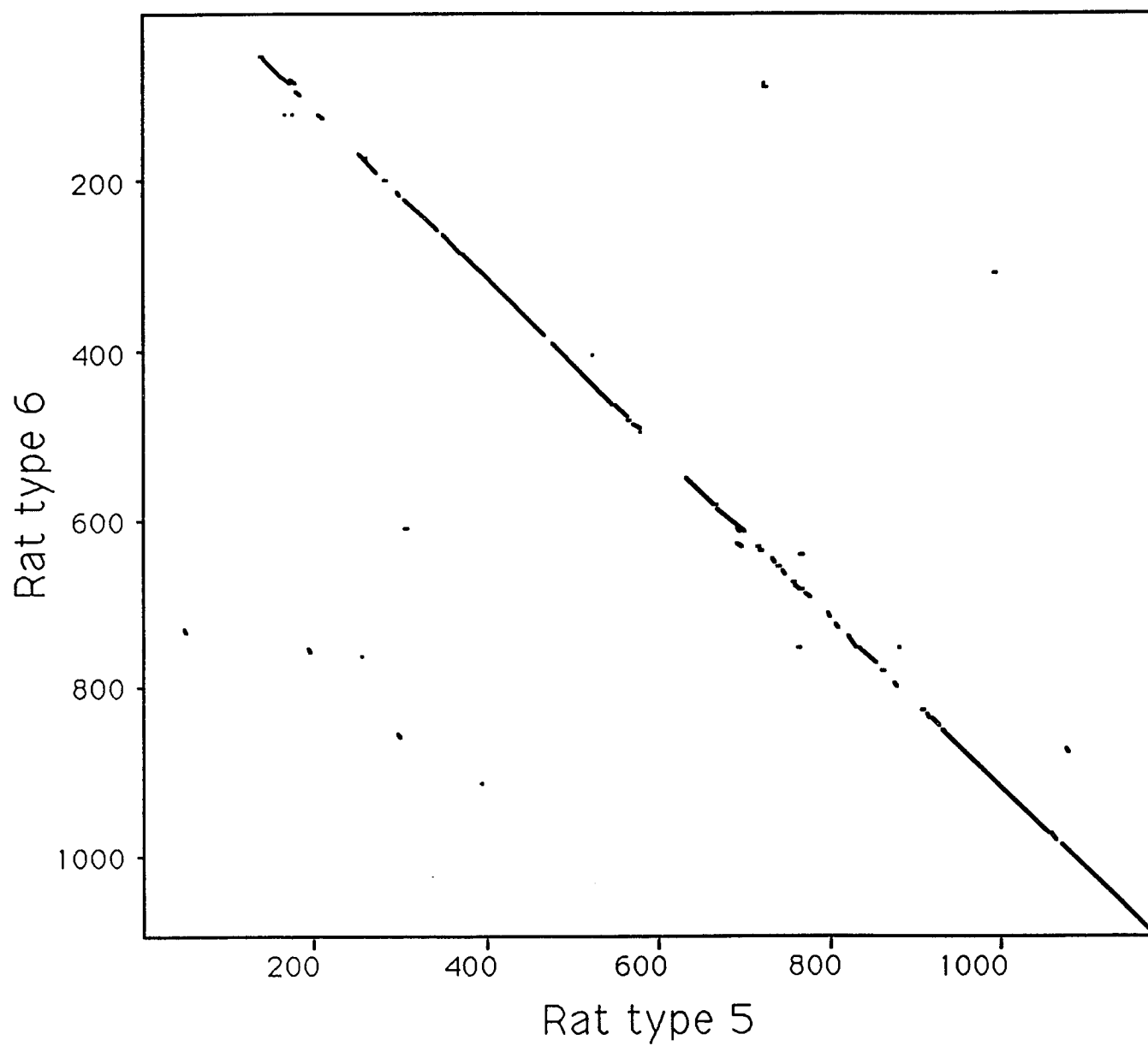
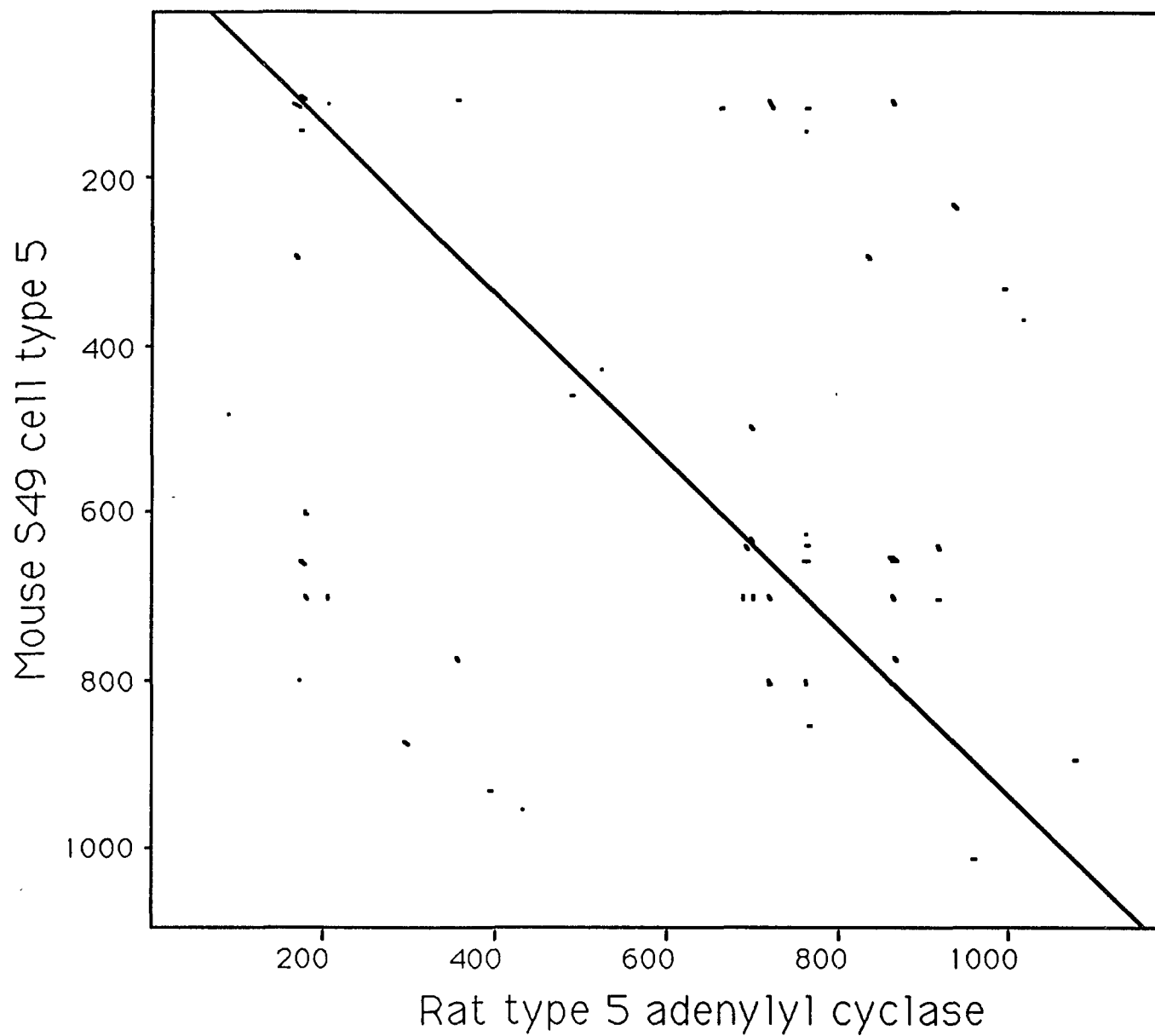


Figure 6-11. Matrix comparison of rat type 5 adenylyl cyclase and mouse S49 lymphoma cell (type 5) adenylyl cyclase deduced amino acid sequences. Comparison conditions are as described for Figure 6-9.

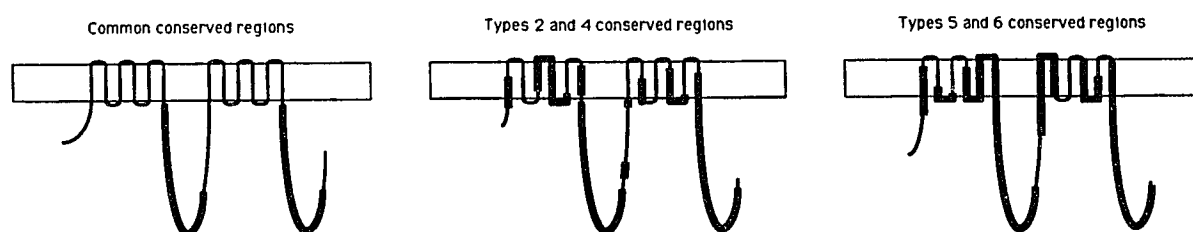


similarity with the rat type 5 and 66% identity and 75% similarity with the rat type 6 adenylyl cyclase in the 1100 amino acid overlap so far determined, and matrix comparison of the rat and mouse type 5 sequences demonstrates this near-identity (Figure 6-11). Thus this S49 cell adenylyl cyclase is clearly the mouse homolog of the rat type 5 adenylyl cyclase.

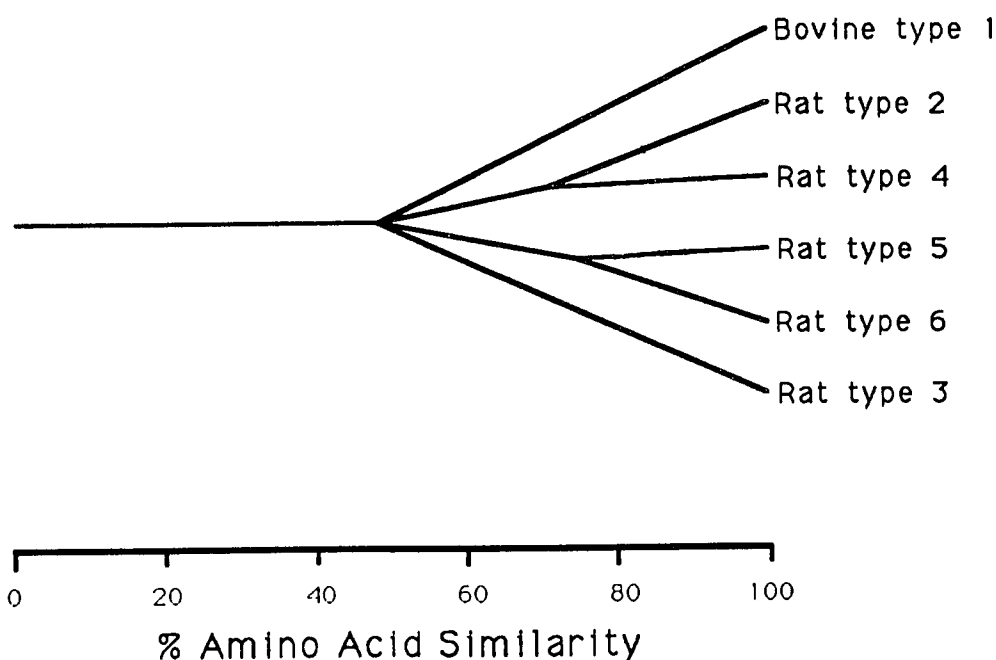
From comparison of the aligned adenylyl cyclase sequences, it is possible to deduce some relationships among the sequences (Figure 6-12). The deduced amino acid sequences of the two rat liver adenylyl cyclases share the highest similarity with each other, and contain lesser similarity with the other adenylyl cyclase sequences. Among these cloned sequences, the liver type 5 and 6 adenylyl cyclases and the type 2 and 4 sequences appear to form two distinct sub-families. Regulation of the enzymes within these sub-families will probably be similar. For the type 2 and 4 adenylyl cyclases, both  $G_s$  stimulation and an unexpected stimulation by  $\beta\gamma$  subunits of G proteins (in the presence of activated  $\alpha_s$  protein) has been demonstrated, while calmodulin was without effect (Tang & Gilman, 1991; Gao & Gilman, 1991; Federman *et al*, 1992). The type 1 adenylyl cyclase is directly stimulated by  $Ca^{2+}$ /calmodulin as well as  $G_s$  (Tang *et al*, 1991), but is slightly inhibited by  $\beta\gamma$ -subunits (Tang & Gilman, 1991). The detailed regulation of the type 5 and 6 adenylyl cyclases remains to be determined (but see below).

Both the type 5 and 6 adenylyl cyclases contain relatively long 5' regions, having more sequence before membrane span 1 than the other four cloned mammalian adenylyl cyclases. This N-terminal region is rather divergent among adenylyl cyclase forms, but

Figure 6-12. Homology relationships among cloned adenylyl cyclases. *Top*: From matrix comparisons such as Figures 6-9 through 11, regions of high similarity (>60%) were mapped onto the predicted structure of the adenylyl cyclase (see Figure 1-1), for all (6.5) cloned mammalian adenylyl cyclases, for types 2 and 4, and for types 5 and 6, as indicated. *Bottom*: From pairwise alignment and comparison of six cloned adenylyl cyclase sequences, the percentage of identical and conservatively substituted residues over the complete sequences was determined for all combinations. From these percent similarity scores, a tree was constructed by iteratively connecting the highest scoring pair of sequences until all sequences were matched with all others. Differences in scores of less than 5% between branches were considered insignificant, so that the sequence families appear to diverge from a single point at 50% similarity.



### Relationships Among Cloned Adenylyl Cyclases



the type 5 and 6 sequences have some similarity to each other throughout this stretch as well. Neither the type 5 and 6 subfamily nor the type 2 and 4 subfamily of adenylyl cyclases contains the unconserved carboxyl terminal tail region found in the types 1 and 3 and in a human brain partial adenylyl cyclase sequence (and in cloned guanylyl cyclases). The type 5 and 6 sequences are truncated even compared to the type 2 and 4 sequences, and have the shortest C-terminal tails of any cloned mammalian adenylyl cyclase, with only a few residues after the last absolutely conserved residue.

#### Prediction of protein kinase phosphorylation sites in adenylyl cyclases

The rat type 5 adenylyl cyclase has a single predicted protein kinase A phosphorylation site at Ser<sup>674</sup> (as does the mouse S49 cell sequence in the equivalent position), while the type 6 has two predicted protein kinase A sites. One is at Ser<sup>671</sup> of the currently-known sequence, which is in the loop between membrane spans 8 and 9, and the other is at Ser<sup>591</sup>. This site is shared between the type 5 and 6 sequences in a short, relatively conserved region immediately before membrane span 7 at the end of the first large intracellular domain (Figure 6-13). Detailed comparison with the other mammalian adenylyl cyclase sequences reveals that this protein kinase A phosphorylation site is not shared with any of the other identified adenylyl cyclases. Types 1 and 3 (and a human brain partial sequence) have only one predicted protein kinase A site, while the type 2 sequence has two sites (one is between membrane spans 8 and 9). The type 4 adenylyl cyclase has no predicted sites. These protein kinase A consensus sites in other adenylyl cyclases are found in widely disparate locations. Functional evidence for lowering of



adenylyl cyclase activity following phosphorylation by protein kinase A in liver and S49 lymphoma cells was presented in Chapter 5. The diverse adenylyl cyclase forms currently isolated may respond diiferentially to protein kinase A phosphorylation. The presence of type 5 adenylyl cyclase in both liver and S49 cells implies that this enzyme may be one which is negatively regulated by protein kinase A. The ability of individually expressed adenylyl cyclases to be regulated by protein kinase phosphorylation may now be determined directly.

Protein kinase C consensus phosphorylation sites are found in many locations throughout all of the adenylyl cyclase sequences, while few are shared among even more-related sequences. The type 5 sequence has 12 putative protein kinase C consensus phosphorylation sites, and the type 6 sequence contains 11 sites (so far). Only 5 are in common locations between types 5 and 6. Of these, only three are also in common with another adenylyl cyclase (two with type 1, and one with a human brain partial sequence). No predicted protein kinase C phosphorylation sites are found in common among all adenylyl cyclase sequences. Nevertheless, adenylyl cyclase activity is known to be enhanced by direct protein kinase C-mediated phosphorylation (Yoshimasa *et al*, 1987; 1988). Such activity enhancement has been noted in the S49 lymphoma cell line (Bell *et al*, 1985), and could be due to phosphorylation of the type 5 adenylyl cyclase protein, which is found in this cell. Differential regulation of the various adenylyl cyclases by protein kinase C-mediated phosphorylation is also possible, and will need to be experimentally addressed as well.

### Expression of adenylyl cyclase mRNAs

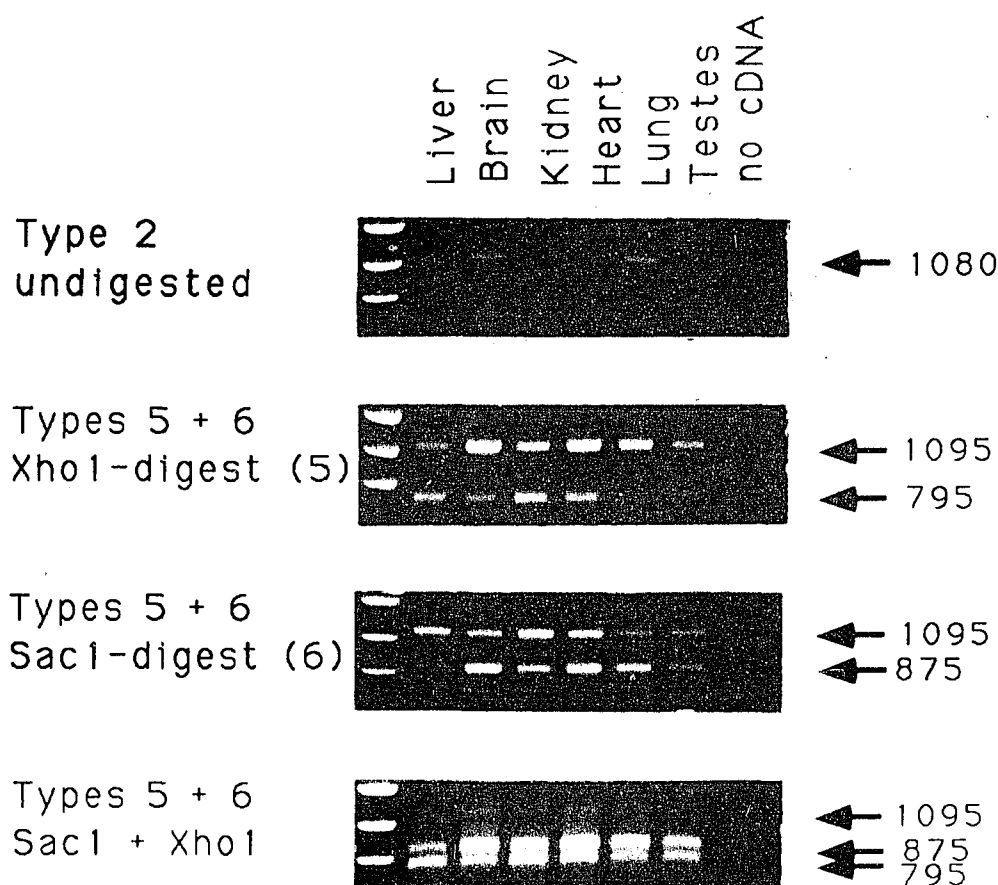
Most of the previously cloned adenylyl cyclases have been shown to have relatively restricted mRNA expression. The type 1 adenylyl cyclase is found in brain (Krupinski *et al*, 1989; Xia *et al*, 1991). The type 2 enzyme is present in brain and lung (Feinstein *et al*, 1991). The type 3 form is present mainly in olfactory neuroepithelium (Bakalyar & Reed, 1990). The type 4 adenylyl cyclase is the only form shown to have a relatively broad tissue distribution (Gao & Gilman, 1991). The distribution of any of the various adenylyl cyclase proteins is entirely unknown at present.

Northern blotting (by Madhavi Ponnappalli) of 15 µg of poly(A)RNAs from various rat tissues performed using 600 bp cRNA probes to the type 5 and 6 adenylyl cyclase 3'-untranslated regions indicates a relatively low level of mRNAs for each in the tissues examined (liver, brain, kidney).

A wider search for adenylyl cyclase mRNA expression was performed by PCR amplification of specific fragments of individual adenylyl cyclases from first-strand cDNAs prepared from several rat tissues. Oligonucleotide primer pairs flanking 1100 bp of sequence encoding membrane spans 7→12 were prepared for the rat types 2, 3 and 4 adenylyl cyclases from the published sequences (Feinstein *et al*, 1991; Bakalyar & Reed, 1990; Gao & Gilman, 1991). Primers made using the bovine type 1 sequence (Krupinski *et al*, 1989) were found to amplify a proper-size band from rat brain, while primers prepared for a human partial adenylyl cyclase sequence (Parma *et al*, 1991) fail to amplify a proper band from rat cDNAs. A single pair of primers was also prepared to amplify the analogous region of both the rat type 5 and 6 adenylyl cyclases. Amplifications were

performed, and the products separated on agarose gels. The reactions shown were all performed in parallel with the same cDNAs. For the type 2 adenylyl cyclase, 50  $\mu$ l of 100  $\mu$ l PCR reactions were loaded on the gel, and the expected 1080 bp fragment was amplified only from rat brain and lung cDNAs (Figure 6-14A). The authenticity of the band was confirmed by digestion into 800 and 300 bp fragments by digestion with *Sca*1 (not shown). For the type 5 and 6 adenylyl cyclases, 15  $\mu$ l of the reactions were loaded. The 1095 bp type 5 and 6 band was observed in all tissue cDNAs examined. To determine the presence of type 5 amplified DNA, the 1095 bp product was digested with *Xho*1 into 795 and 300 bp bands (Figure 6-14B), while the presence of type 6 amplified DNA was determined by digestion with *Sac*1 into 875 and 220 bp bands (Figure 6-14C). Digestion of the 1095 bp DNA with both *Xho*1 and *Sac*1 resulted in the loss of 1095 bp product and the appearance of both expected digestion products (Figure 6-14D). This complete digestion provides weak evidence that no closely-related "type 7" adenylyl cyclase sequence was also amplified. The relative intensities of the types 5 and 6 amplified bands presumably reflect the relative ratios of their mRNAs in these tissues, although the linearity of the PCR reaction under these conditions has not been verified. By both Northern blotting and by PCR amplification of specific fragments from tissue cDNAs, it appears that both the type 5 and 6 adenylyl cyclases are widely expressed in diverse rat tissues. The reported widespread expression of the recently-cloned rat type 4 adenylyl cyclase (Gao & Gilman, 1991) has also been verified by this method (data not shown).

Figure 6-14. Distribution of type 2 and types 5 and 6 adenylyl cyclase mRNAs by PCR amplification of first-strand cDNAs. Poly(A) RNAs were prepared from several rat tissues, oligo(dT)-primed first-strand cDNAs were synthesized from 1  $\mu$ g of poly(A) RNA. Pairs of oligonucleotide primers flanking regions encoding membrane spanning regions 7 $\rightarrow$ 12 were prepared from the published rat type 2 adenylyl cyclase sequence (Feinstein *et al*, 1991), and from the rat type 5 and 6 sequences. Primers were used to amplify 1100 bp product DNA bands by PCR from various rat tissue cDNAs (equivalent to 10 ng of poly(A) RNAs). For the type 2 reactions, 50  $\mu$ l was separated on 1% agarose gel to visualize the 1080 bp product band (*Panel A*). Digestion of the remainder of the crude reaction mixture with Sca1 led to digestion of this 1080 bp band (not shown). For types 5 and 6, 15  $\mu$ l of the reaction was subjected to digestion with Xho1 which cleaves only the type 5 DNA into 795 and 300 bp bands (*Panel B*), digestion with Sac1 which cleaves only the type 6 DNA into 875 and 220 bp bands (*Panel C*), or digestion with both enzymes together (*Panel D*).



### Expression of type 5 adenylyl cyclase cDNA

The rat type 5 adenylyl cyclase has been used for expression studies (by Jianqiang Chen). This rat type 5 clone was reconstructed from both cloned fragments and PCR-amplified fragments from liver cDNA. This construct was inserted into the expression vector pcDNA I (Invitrogen) for expression from the CMV promoter. Human kidney 293 fibroblasts were transfected with with pcDNA-AC5 DNA in the presence of DEAE-Dextran, and subsequently assayed for transient expression of the cloned adenylyl cyclase. Cells transfected with the type 5 expression vector express 3-5 times the basal (unstimulated) adenylyl cyclase activity of cells transfected with vector alone or that are untreated. This increased activity is also evident in the presence of forskolin, GTP $\gamma$ S, or isoproterenol and GTP, indicating that the type 5 adenylyl cyclase is a functional adenylyl cyclase that can be stimulated by hormones through G<sub>s</sub>. The GTP $\gamma$ S-stimulated activity of the type 5 enzyme is not further enhanced by addition of 100 nM purified  $\beta\gamma$ -subunits of G proteins, while expressed type 2 enzyme is stimulated 3-fold further than with GTP $\gamma$ S alone. Examination of further regulatory properties of the type 5 clone, as well as further cloning efforts to isolate the extreme 5' end of the type 6 clone and the expression of the type 6 adenylyl cyclase, are in progress.

### Summary

The recent cloning of a bovine brain calmodulin-stimulated (type 1) adenylyl cyclase (Krupinski *et al*, 1989) has allowed the further isolation of several distinct adenylyl cyclase forms. The diversity of mammalian G<sub>s</sub>-regulated adenylyl cyclases

which has so far been revealed far exceeds expectations based on functional distinctions among subtypes of the enzyme (Neer, 1978b).

The initially-cloned type 1 sequence has been functionally expressed, and shown to encode a  $G_s$ -, forskolin-, and calmodulin-stimulated adenylyl cyclase (Krupinski *et al*, 1989; Tang *et al*, 1991). The enzyme has a very high basal level of activity, and can be substantially inhibited by "P"-site agonists and by  $\beta\gamma$ -subunits of G proteins (Tang *et al*, 1991), but is not at all inhibited by  $G_i$  or  $G_o$   $\alpha$ -subunits when stimulated with  $G_s$  or forskolin (Linder *et al*, 1990). The distribution of the mRNA for this enzyme has been mapped in rat brain using probes from the bovine sequence, and was found to be highly expressed in many brain regions associated with learning (Xia *et al*, 1991). This was expected since the *Drosophila* associative learning mutation *rutabaga* had been associated with loss of calmodulin-sensitive adenylyl cyclase activity in head homogenates (Livingstone *et al*, 1984; Livingstone, 1985). The wild-type and mutant *rutabaga* gene products have recently been cloned by hybridization to the bovine type 1 clone, and shown to encode a calmodulin-sensitive adenylyl cyclase very similar to the bovine type 1 enzyme (Levin *et al*, 1992). The *rutabaga* phenotype results from a point mutation which converts an absolutely conserved glycine to arginine in the second conserved domain of the enzyme.

An adenylyl cyclase cDNA (type 3) isolated from rat olfactory neuroepithelium library appears to encode a sensory form of adenylyl cyclase (Bakalyar & Reed, 1990). Expression studies indicate that this enzyme has a very low basal level of activity, and is stimulated by forskolin and NaF (through  $G_s$ ). The mRNA for this enzyme is

expressed at high levels in the olfactory neuroepithelium, but is not apparent in several other tissues. The stimulated activity of the type 3 adenylyl cyclase was not affected by addition of purified  $\beta\gamma$ -subunits of G proteins (Tang & Gilman, 1991). Calmodulin sensitivity has not been tested, but olfactory adenylyl cyclase activity is known to have a calmodulin-sensitive component (Anholt & Rivers, 1990).

Two additional cloned and expressed adenylyl cyclases have been reported, from rat brain (type 2) and from rat testis (type 4) (Feinstein *et al*, 1991; Gao & Gilman, 1991). These two sequences share much greater similarity with each other than with the type 1 or type 3 sequences, and appear to form a distinct subfamily of adenylyl cyclases (see Figure 6-12). The type 2 mRNA is expressed mainly in brain, with significant levels also in lung, while the type 4 mRNA appears to be quite widely expressed in rat tissues. Type 2 and type 4 enzymes are stimulated by forskolin and  $G_s$ , but not by calmodulin. An unexpected finding was that addition of purified  $\beta\gamma$ -subunits of G proteins to type 2 and type 4 adenylyl cyclases leads to several-fold stimulation of activity. This  $\beta\gamma$  stimulation is conditional, since it occurs in the presence of GTP $\gamma$ S-activated  $G_s\alpha$  but not with unstimulated adenylyl cyclase (Tang & Gilman, 1991; Gao & Gilman, 1991). For the type 2 enzyme, GTP $\gamma$ S-activated  $G_i\alpha$  was unable to support this  $\beta\gamma$  stimulation, and also had no effect on basal adenylyl cyclase activity. Thus type 2 adenylyl cyclase (and probably type 4 also) does not appear to be inhibitable by  $G_i$ , either by  $\alpha$  or  $\beta\gamma$ -subunits. A more recent paper reports stimulation of expressed type 2 adenylyl cyclase activity by hormones which activate  $G_i$  and normally inhibit adenylyl cyclase activity (Federman *et al*, 1992). This stimulation requires the simultaneous stimulation by hormones which

activate adenylyl cyclase through  $G_s$ . This indicates that the conditional hormonal stimulation of type 2 and 4 adenylyl cyclases by hormones which activate  $G_i$  (and normally inhibit adenylyl cyclase activity in most tissues) is a normal property of the type 2 protein and not an artefact of adding purified G protein subunits to the system. A partial human type 2 adenylyl cyclase clone has also been isolated from brain cDNA (Stengel *et al*, 1992).

Another distinct adenylyl cyclase has been identified by cloning of a partial cDNA from a human brain library (Parma *et al*, 1991). This sequence contains the second set of transmembrane spans and the second conserved domain, and appears to be quite distinct from the type 1, type 3, and type 2 and 4 adenylyl cyclase subfamilies.

This chapter documents the identification and cloning of two additional forms of adenylyl cyclase from rat liver and kidney, which we have tentatively called types 5 and 6. These two sequences encode proteins which are 75% similar with each other but 50% similar with the previously-cloned sequences, indicating that like the type 2 and 4 adenylyl cyclases, the type 5 and 6 adenylyl cyclases form a distinct subfamily. PCR amplification of first-strand cDNA using subtype-specific primers indicates that mRNA encoding each of these enzymes appears to be widely distributed in rat tissues. Expression of the type 5 enzyme produces an adenylyl cyclase activity which is stimulated by forskolin, GTP $\gamma$ S and hormones plus GTP. Stimulation of the type 5 enzyme by GTP $\gamma$ S is not enhanced by addition of purified  $\beta\gamma$ -subunits, while activity of expressed type 2 enzyme is markedly enhanced. Therefore, the type 5 and 6 adenylyl cyclase subfamily appears to have functional properties and distribution consistent with

adenylyl cyclase activity measured in most tissues (except brain). While calmodulin sensitivity has not yet been directly tested, since liver and most tissues where type 5 and 6 enzymes appear to be found do not exhibit such sensitivity, it is likely that this subfamily is unresponsive to stimulation by calmodulin.

The type 1 and type 3 adenylyl cyclases appear to be highly specialized forms which perform particular roles in defined regions of the body. The type 2/4 subfamily and the type 5/6 subfamily, however, appear to have members located throughout many tissues. The finding that there are (at least) two distinct subfamilies of somatic calmodulin-insensitive (presumably) adenylyl cyclases is quite unexpected. The similarity of type 2 and 4 sequences appears to correlate well with similar regulation of the enzymes. Expression of the type 6 enzyme will be required to test whether its regulation is also as similar to that of the type 5 enzyme. The distinct role each subfamily plays in regulation of cAMP synthesis is still unknown, but the recent discovery of conditional  $\beta\gamma$ -stimulation of the type 2/4 adenylyl cyclase subfamily indicates that regulation of this enzyme may be far more complex than had been previously imagined. Distinct regulation of individual adenylyl cyclases may also occur by various protein kinases, "P"-site agonists, and inhibitory G proteins. These distinct subfamilies of adenylyl cyclases will probably display quite dissimilar regulatory properties, which together make up the complex regulation of the activity seen in a cell.

## CHAPTER 7

### CONCLUSIONS

Treatment of tissues and cells with hormones which activate adenylyl cyclase induces an overall loss of the ability of adenylyl cyclase to be subsequently stimulated. It has been well-known for many years that the desensitization of the adenylyl cyclase system results from multiple independent events (reviewed in Harden, 1983; Clark, 1986; Sibley & Lefkowitz, 1985; 1988). Desensitization responses were initially subdivided into two classes based on whether response to hormones in addition to that used to induce the desensitization was lost (Su *et al.* 1979; 1980). More recently, it has become clear that each of these broad classes of desensitization response may be composed of several distinct mechanisms, as outlined in Chapter 1 (see Figure 1-2).

Treatment of a cell with a hormone leads to a complex series of modulations with the effect of adjusting the system's sensitivity or gain to match the ambient signal level. Analysis of these modulations reveals that homologous desensitization is both faster and of greater extent than heterologous desensitization, but requires higher agonist concentrations (Clark *et al.*, 1988; Hausdorff *et al.*, 1989). Prolonged signalling at even very low levels of hormone lead to changes that are generally heterologous, since little cAMP is required to fully activate protein kinase A. Thus the homologous desensitization response is the cell's primary line of defense against acute overstimulation by a single hormone, but is not necessarily the major physiological response (except perhaps at neural synapses). A variety of smaller cAMP-dependent modulations (heterologous

desensitization) are called upon most often to reduce signaling through both homologous and heterologous receptors. One important aspect of heterologous desensitization which I have not explicitly explored is the concurrent hormonal modulation of the sensitivity of signaling through other systems, such as phospholipase C or inhibition of adenylyl cyclase. Heterologous modulations of signaling systems thus play important roles in signal integration as well as preventing overstimulation (Reithmann *et al*, 1991).

Despite great increases in our understanding of which components of the adenylyl cyclase system may serve as targets for modulation (all of them) and of certain of the mechanisms underlying desensitization of individual components (particularly the receptors), great uncertainties remain. It has been clear for many years that there are major variations in the desensitization response among diverse cell types, and even between different hormones in the same cell. While the mechanisms for modulation individual components of the adenylyl cyclase system appear remarkably similar even across great phylogenetic distances (ie., receptor kinases in *Dictyostelium* and *Drosophila*), differing cells appear to choose to use only certain modes of regulation from the menu of available mechanisms. Why some cell types appear to exhibit only some of the possible forms of desensitization response is an entirely open question (although forms causing lesser effects on activity may have been obscured by larger effects in any given study). Analysis of the relative contributions of the various components of desensitization to the overall desensitization response, and the ability to manipulate these contributions by expression of normal or mutated components of the signaling pathway, will allow a better understanding of this variation in desensitization among tissues. Two examples of such apparent variations in desensitization responses are given below.

In many cell types, desensitization caused by  $\beta$ -agonists appears to be purely homologous and often results in complete loss of stimulation of adenylyl cyclase. However, in the same cell, a peptide hormone may induce both homologous and heterologous desensitizations, and yet never produce a total loss of hormone stimulation of adenylyl cyclase. For example, in studies of cultured rat hepatocytes, treatment with glucagon induces extensive loss of adenylyl cyclase stimulation by both glucagon and  $\beta$ -adrenergic agonists (homologous and heterologous desensitization).  $\beta$ -agonist treatment results in near total loss of  $\beta$ -adrenergic stimulation of adenylyl cyclase, but little loss of glucagon stimulation (homologous desensitization) (Gurr & Ruh, 1980; Noda *et al*, 1984). Both hormones activate adenylyl cyclase to produce intracellular cAMP, yet one induces almost no heterologous response. Glucagon can induce a much greater (3-5-fold) maximal stimulation of adenylyl cyclase than activation of the  $\beta$ -adrenergic receptor in these cells, so one potential explanation is that the higher cAMP accumulation induced by glucagon treatment is critical for the development of a heterologous desensitization response. However, this appears contradictory to the finding that only a small fraction of the total adenylyl cyclase stimulatory response is required to fully activate the protein kinase A within the cell and thereby produce the heterologous desensitization (Hausdorff *et al*, 1989). The possible role of the maximal stimulation of adenylyl cyclase through a given receptor determining the type of desensitization response can be explicitly tested by expressing varying amounts of a cloned receptor in a cell and correlating the expressed receptor density with the components of the desensitization response that are observed (homologous at low density and heterologous at high density ?). A second possibility is that some receptors themselves are better substrates for phosphorylation by protein kinase

A or BARK-like receptor kinases, leading to faster or more extensive receptor uncoupling, or are more responsive to the mechanisms responsible for receptor internalization. Few such comparisons between distinct receptors have been made, although receptor mutation and expression studies may illuminate these differences as well.

There appear to be at least two distinct cAMP-dependent mechanisms underlying distinct components of heterologous desensitization, phosphorylation of receptor proteins and phosphorylation of adenylyl cyclase. In the MDCK cell line, glucagon and 8-Br-cAMP induce a heterologous desensitization at the level of receptors, but no direct effect on adenylyl cyclase was apparent. Addition of exogenous purified  $G_s$  to glucagon-treated MDCK cell membranes is able to restore normal levels of NaF-stimulation of adenylyl cyclase, but stimulation through glucagon and  $PGE_1$  receptors remained desensitized, due to a block at the level of the receptors (Rich *et al*, 1984). In chick hepatocytes, however, glucagon and 8-Br-cAMP treatment appears to induce a heterologous desensitization due to modulation of the adenylyl cyclase directly, but without an apparent cAMP-dependent effect on glucagon or VIP receptor coupling to  $G_s$  (Chapter 5). Exogenous purified  $G_s$  added to glucagon-treated hepatocyte membranes is capable of restoring an equivalent portion of  $G_s$ -mediated stimulation of adenylyl cyclase both by NaF and by hormones, but a cAMP-dependent loss of adenylyl cyclase stimulation cannot be overcome (Chapter 5). Thus in two cell types, the major cAMP-dependent steps in heterologous desensitization appear to be completely distinct. Both cells certainly contain protein kinase A, so why is one phosphorylation substrate apparently preferred over another in these different cells? The recent cloning of several distinct mammalian  $G_s$ -stimulated adenylyl cyclases raises the possibility that some forms are regulated by protein kinase A while others are not.

This can now be experimentally addressed by expression of individual adenylyl cyclase cDNAs. A second explanation is that the glucagon receptor in chick liver and canine kidney (MDCK cells) differ in their susceptibility to regulation by protein kinase A. Cloning of the glucagon receptor cDNA will allow such direct comparisons as well.

From these examples (many others are also possible), it is clear that the regulation of the adenylyl cyclase system is not as simple as presented in Chapter 1, with equivalent receptors activating equivalent  $G_s$  activating equivalent adenylyl cyclase in all tissues. Tissue variations in the expression and regulation of the components of the adenylyl cyclase system may have a profound effects on the components of the desensitization response, and thus underly the tissue-specific character of this signalling pathway.

### Regulation of receptors

Regulation of receptor proteins in homologous desensitization has been proposed to result from the agonist-dependent phosphorylation of the receptor by a receptor kinase and the subsequent association of an arrestin protein with the phosphorylated receptor, which uncouples the receptor from further activation of  $G_s$  (Benovic *et al*, 1986a; 1986b; 1987a; 1989; Lohse *et al*, 1990). Three receptor kinases have been identified by cloning: rhodopsin kinase which uncouples rhodopsin from transducin in the visual system (Lorenz *et al*, 1991), and two forms of so-called " $\beta$ -adrenergic receptor kinase" or BARK (Benovic *et al*, 1989; 1991). Other members of this family of kinases may also exist, such as the so-called "muscarinic receptor kinase" activity purified from brain (Haga & Haga, 1989; 1992). Four distinct receptor kinase related sequences have recently been identified in *Drosophila* (Cassill *et al*, 1991). Mutation studies and mapping of

phosphorylation of the purified  $\beta$ -adrenergic receptor protein have defined a region of the C-terminal tail of the receptor as a site for multiple phosphorylations by BARKs (Dohlman *et al*, 1988; Liggett *et al*, 1989; Hausdorff *et al*, 1989).

The recent cloning of several adenylyl cyclase-coupled receptors of a class distinct from that of the previously known rhodopsin/ $\beta$ -adrenergic receptor class (Dohlman *et al*, 1987) has striking implications (see Table 1-1). Sites mapped to the cytoplasmic face of the intracellular loop connecting membrane spans 5 and 6 of the  $\beta$ -adrenergic receptor which appear to be required for receptor stimulation of  $G_s$  are not conserved in this second class of receptors. Therefore, they may stimulate  $G_s$  in some novel way. Similarly, there is no current evidence that any of the identified receptor kinases are capable of recognizing or phosphorylating members of this second class of  $G_s$ -coupled receptor, nor that any arrestins can interfere with their coupling to  $G_s$ .

One of this new class of receptors is the heart secretin receptor. Glucagon and secretin are related peptide hormones, so the glucagon receptor may also be a member of this new class of receptor proteins. The observed regulation of the chick hepatocyte glucagon receptor by rapid glucagon-stimulated uncoupling from  $G_s$  is consistent with a receptor kinase mechanism. In the absence of a direct molecular probe for isolating the receptor protein to determine its phosphorylation state, or a cloned cDNA to mutate and express, it is not currently possible to unequivocally establish the mechanism for this regulation in the hepatocytes.

Glucagon-induced homologous desensitization in isolated rat hepatocytes also appears to be due to the rapid uncoupling of the glucagon receptor from  $G_s$  (Heyworth & Houslay, 1983). No associated changes in glucagon receptor binding activity measured

at a single probe concentration were reported, but binding was examined only within several minutes of agonist treatment. However, this system has several features which make it unusual compared to the chick hepatocyte system. Foremost is the finding that activation of phospholipase C or protein kinase C, but not elevation of cAMP, appears to induce desensitization identical to that induced by glucagon (Heyworth & Houslay, 1983; Heyworth *et al*, 1984b; 1985; Murphy *et al*, 1987; 1989; Newlands & Houslay, 1991). Nevertheless, inhibition of cAMP phosphodiesterase during and following glucagon treatment prevents recovery from desensitization (Murphy & Houslay, 1988). Prior treatment of hepatocytes with pertussis toxin prevented the development of glucagon-induced desensitization (Heyworth *et al*, 1984a), and this homologous desensitization was reported to be associated with phosphorylation and inactivation of  $G_{i2}$ - $\alpha$  (Pyne *et al*, 1989), but not due to this modification (Murphy *et al*, 1989).

Longer treatment with glucagon induces loss of measurable receptor binding sites from the plasma membranes. Receptors within the cell (endosomes) could not be reliably detected due to relatively high non-specific binding of the [ $^{125}$ I]MIG probe compared with the apparently low level and affinity of "internalized" binding sites. However, glucagon receptor binding activity has been reported in intracellular vesicle fractions from rat liver (Lipson *et al*, 1986; de Diego *et al*, 1987).

In the  $\beta$ -adrenergic system, receptor internalization can be further subdivided into an initial sequestration step and later down-regulation of the receptor protein. Sequestration results from internalization of the receptor to a pool which appears to be close to and exchange rapidly with the plasma membrane, allowing rapid recovery upon hormone withdrawal. Sequestered  $\beta$ -adrenergic receptors can be measured using

membrane permeant receptor probes (Staehelin & Hertel, 1983) and can be recoupled to  $G_s$  by polyethylene glycol treatment of intact cells, which appears to induced fusion of the plasma membrane with the "sequestration vesicle" bearing the sequestered receptors (Strasser & Lefkowitz, 1985). Down regulation involves a further internalization of the receptors leading to destruction of the receptor protein, so that recovery is slow and requires new protein synthesis (Doss *et al*, 1981). Receptor sequestration and down-regulation appear to be distinct internalization events rather than two steps along the same pathway (Rich & Iyengar, 1989).

Measurement of glucagon receptors is made using an agonist probe, [ $^{125}$ I]MIG, and so the amount of binding activity varies with the affinity state (coupled to  $G_s$  or uncoupled) of the receptor. It is not possible to determine the maximal glucagon receptor number, since some receptors of low affinity may well be missed. This peptide ligand is also not permeable to cellular membranes, and so in the absence of additional specific probes for the glucagon receptor (permeant antagonist ligand or antisera), it is not possible to directly distinguish receptor sequestration from down-regulation by binding assays as can be done for the  $\beta$ -adrenergic receptor (Staehelin & Hertel, 1983). Using analysis of the recovery rate following various lengths of glucagon treatment, and sensitivity of recovery to various RNA or protein synthesis inhibitors, such a distinction could probably be made in this system. Another method for determining the extent of down-regulation of the receptor protein in response to agonist treatment would be to characterize the turnover rate of the protein under basal conditions and following agonist treatment. Cells can be labeled with "dense" amino acids containing  $^2\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ , and new and old receptor proteins separated by density using centrifugation in sucrose gradients. The

proportion of the receptor population in "heavy" and "light" fractions as a function of time following addition of dense amino acids is a measure of the synthesis and destruction of the receptor protein. By comparison of these rates under basal and hormone-stimulated conditions, the effect of hormone on the balance of receptor synthesis and destruction can be directly determined. This technique has been to determine the effect of agonists on the turnover rate of the  $\beta$ -adrenergic receptor in astrocytoma cells (Waldo *et al*, 1984), and of the insulin receptor in chick hepatocytes (Krupp & Lane, 1981).

### Regulation of G proteins

Glucagon treatment of chick hepatocytes induces a rather rapid (<30 min) loss of NaF-stimulated adenylyl cyclase activity which is associated with loss of  $G_s$  activity which can be extracted from the membrane using detergent. This effect is also rapidly reversed upon removal of glucagon from the cell surface. Addition of purified  $G_s$  to desensitized hepatocyte membranes is able to partially restore normal stimulation of adenylyl cyclase by NaF and by hormones (glucagon, VIP). Direct measurement of  $G_s$  protein levels by quantitative immunoblotting failed to detect a significant loss of  $G_s\alpha$  protein from the cell membranes. It is not clear that this is due to there being no changes in  $G_s$  protein levels, changes in  $G_s$  distribution within domains of or associated with the plasma membranes leading to reduced coupling to receptors and adenylyl cyclase, or simply difficulties with the anti-bovine antiserum in recognizing chicken  $G_s$ .

The activity of the  $G_s$  protein has been observed to be reduced following hormone treatment in a variety of systems.  $PGE_1$  treatment has been reported to result in reduced cholera toxin substrate  $G_s$  protein and 50% reduced  $G_s$  activity in rat liver (Garrity *et al*,

1983), while PGE<sub>1</sub> treatment of human fibroblasts induces a 40% loss of G<sub>s</sub> activity (Kassis & Fishman, 1982). Treatment of rats (Kirchick *et al*, 1983) and rabbits (Jena & Abramowitz, 1989) with hCG results in loss of G<sub>s</sub> activity in corpus luteum. Conversely, "desensitization" in adipocytes with an adenylyl cyclase inhibitory hormone (adenosine) has been reported to lead to 30% increased G<sub>s</sub> activity and increased levels of cholera toxin substrate (Parsons & Stiles, 1987) as well as decreased levels of G<sub>i</sub> (Longabaugh *et al*, 1989; Green *et al*, 1992).

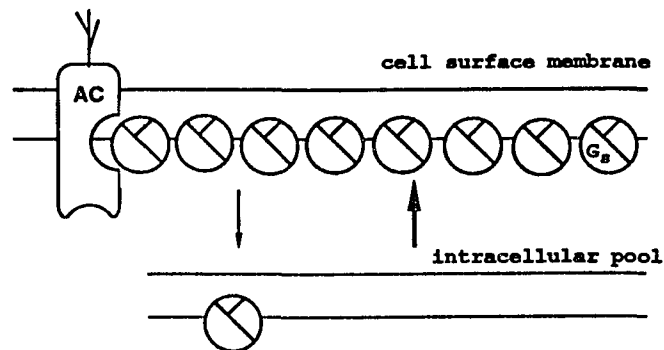
Changes in labeling of G<sub>s</sub>α proteins with cholera or pertussis toxins is suggestive of alterations in protein levels, but this labeling is not necessarily linear. Direct measurement of protein levels by quantitative immunoblotting is a much more powerful method. This technique has been used to document changes in G protein subunit levels in response to steroid hormones (Chang & Bourne, 1987; Ros *et al*, 1988), ethanol treatment (Mochly-Rosen *et al*, 1988), and depolarization of neuronal cells (Luetje *et al*, 1988).

G<sub>s</sub> and G<sub>i</sub> proteins do not appear to be substrates for protein kinase A or protein kinase C phosphorylation (Figure 5-4; Katada *et al*, 1985; Carlson *et al*, 1989; Lounsbury *et al*, 1991). Hormonal regulation of other rapid and reversible post-translational protein modifications which could be responsible for the reduced activity of G<sub>s</sub> in glucagon-treated cell membranes are not currently known.

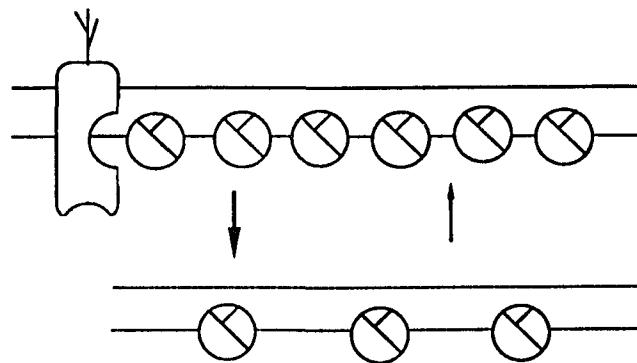
The lack of credible post-translational modifications which could be responsible for functional alterations in G<sub>s</sub> activity lead me to believe that trafficking of the G<sub>s</sub> protein among intracellular membrane pools is the likely cause of this reduced NaF-stimulated adenylyl cyclase activity. A model for this trafficking is shown in Figure 7-1. Proof of

Figure 7-1. Model of the effects of  $G_s$  translocation on the ability of hormones to activate adenylyl cyclase.  $G_s$  protein is proposed to exist in two pools, one in the plasma membrane and available for coupling receptors to adenylyl cyclase, the other sequestered in a location inaccessible to receptors and adenylyl cyclase (either within the cell or in a domain of the plasma membrane). Hormones which activate  $G_s$  induce a shift of  $G_s$  from the surface pool to the sequestered pool. A second challenge with hormone will activate less  $G_s$  per given dose and time, since less  $G_s$  is available to couple to the receptors.

#### Naive state



#### Heterologously desensitized state



this hypothetical mechanism will require not only demonstration of cycling of subunits of the  $G_s$  protein or  $G_s$  activity between distinct pools of coupled (plasma membrane) and uncoupled  $G_s$ , but also a mechanism for agonist-promoted regulation of the redistribution of the G proteins between the pools.

Intracellular  $G_i\alpha$  subunits were detected in neutrophils by Retrosen *et al* (1988).  $\beta$ -Adrenergic stimulation of platelets was reported to induce a redistribution of  $G_i$  to an intracellular pool (Crouch *et al*, 1989), and recently Haraguchi & Rodbell (1990) described  $\beta$ -adrenergic-induced redistribution of  $G_s$  to an intracellular vesicles in adipocytes. These studies are suggestive that G protein subunits may be found in membranes other than the plasma membrane, and that the shuttling of subunits between intracellular and plasma membrane pools may be under hormonal control (reviewed by Milligan & Green, 1991; Reithmann *et al*, 1991). An understanding of the mechanisms for controlling such a system may come from analysis of mutations of the G protein subunits to determine what sequences control the localization of the proteins.

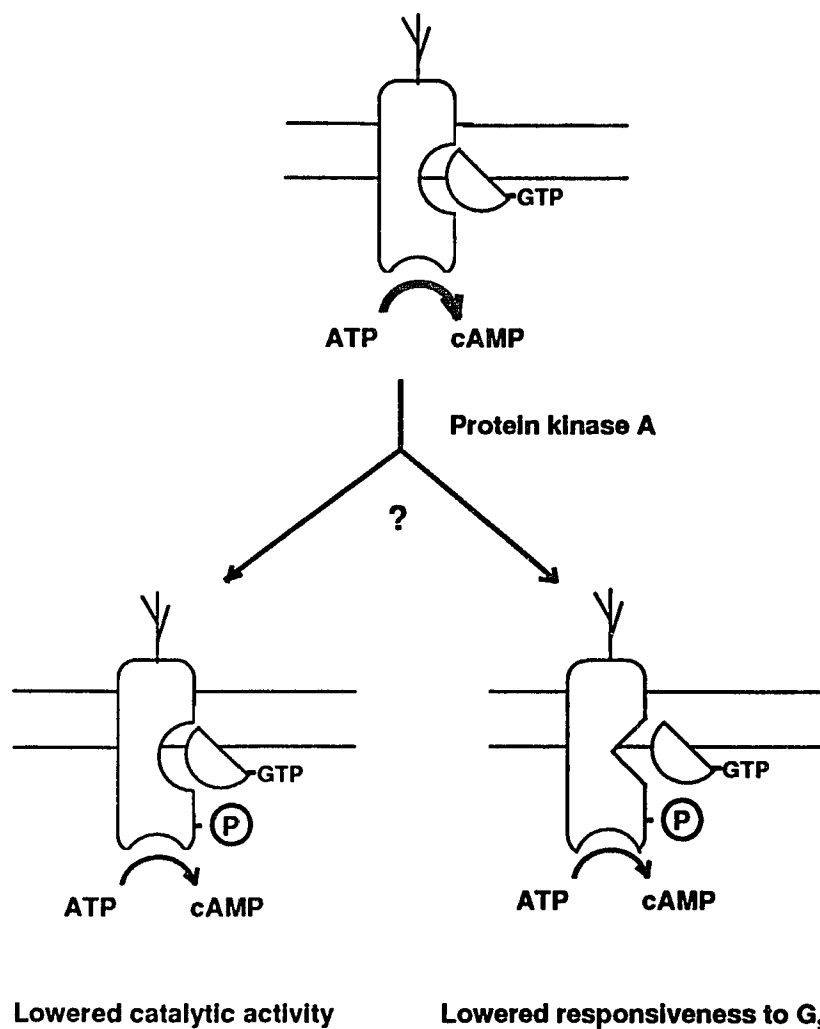
#### Regulation of adenylyl cyclases

Adenylyl cyclase itself appears to be another locus for alteration in heterologous desensitization. Chick hepatocytes treated with 8-Br-cAMP exhibit a partial desensitization of hormone- and NaF-stimulated adenylyl cyclase activity compared to glucagon treatment. This cAMP-induced desensitization cannot be overcome by addition of exogenous  $G_s$ , indicating it is not due to receptor or  $G_s$  phosphorylation. Further, this effect can be produced by treatment of naive membranes from chick hepatocytes or *kin* S49 lymphoma cells with purified protein kinase A and ATP (Chapter 5). Kunkel *et al*

(1988) have also treated S49 cell membranes with protein kinase A and ATP and reported a loss of forskolin-stimulated adenylyl cyclase activity in *kin*<sup>-</sup> but not wild-type cell membranes. Attramadal *et al* (1988) have characterized glucagon-induced *in vitro* desensitization of membranes from cultured rat Sertoli cells, and shown that cAMP treatment of untreated membranes or addition of protein kinase A and ATP to untreated membranes produces a loss of hormone- and NaF-stimulated adenylyl cyclase activity. In an unreviewed chapter, Yoshimasa *et al* (1988) reported the purification of a 150 kDa form of adenylyl cyclase from bovine striatum using forskolin affinity chromatography, and demonstrated its direct phosphorylation by protein kinase A as well as protein kinase C. Upon reconstitution into lipid vesicles, protein kinase A-phosphorylated adenylyl cyclase preparation had reduced activity, while protein kinase C-phosphorylated adenylyl cyclase had elevated activity compared to untreated enzyme.

Phosphorylation of adenylyl cyclase by protein kinase A could reduce the activity of the enzyme by two distinct mechanisms: by reducing the catalytic rate directly, or by reducing the ability of the enzyme to be activated by G<sub>s</sub> (Figure 7-2). Yamashita *et al* (1989) reported an isoproterenol-induced heterologous desensitization of adenylyl cyclase in reticulocytes. This effect could be mimicked by treatment with a cAMP analog. These workers examined the cause of the desensitization by measuring the dissociation rate of activated G<sub>s</sub> from adenylyl cyclase, and found that the G<sub>s</sub>-adenylyl cyclase complex from the desensitized cell membranes dissociated faster (was less stable) than that from untreated cell membranes. Therefore, it seems likely that this decreased stability may be a likely mechanism for the reduced adenylyl cyclase activity observed following adenylyl cyclase phosphorylation.

Figure 7-2. Model of the effects of protein kinase A phosphorylation of adenylyl cyclase on the activity of the enzyme. 1] Phosphorylation of adenylyl cyclase by protein kinase A could reduce the basal and stimulated catalytic rate of the enzyme directly. 2] Phosphorylation could reduce the affinity of adenylyl cyclase for activated  $G_s$ , thereby reducing its ability to be stimulated by a given concentration of  $G_s$ .



I had initially hoped to purify adenylyl cyclase from liver in order to directly determine whether it is a protein kinase A substrate *in vitro* and to determine the functional consequences of such phosphorylation. Together with John Herberg, I purified porcine liver adenylyl cyclase to apparent homogeneity using forskolin affinity chromatography, wheat germ lectin affinity chromatography, and mono-Q FPLC chromatography (RTP, JH and RI, unpublished). A 135 kDa protein was the predominant band observed in purified preparations, which could be crosslinked to [ $\alpha$ - $^{32}$ P]ATP using ultraviolet light. The enzyme has an estimated turnover number of greater than 10,000/min, which is ten-fold more than that measured for purified brain calmodulin-sensitive adenylyl cyclase (Smigel, 1986). However, the purification suffered from such low yield that insufficient purified protein could be accumulated for reconstitution or phosphorylation studies. This was due mainly to very low binding of the enzyme in detergent extracts to the forskolin affinity matrix in the first step, as well as the low basal adenylyl cyclase activity present in the liver. Purification of adenylyl cyclase from brain by the same techniques, on the other hand, was relatively straightforward since a much higher fraction of activity bound to the forskolin matrix (Pfeuffer *et al*, 1985b; Smigel, 1986; RTP, JH and RI, unpublished). Indeed, Mollner & Pfeuffer (1988; 1991) have shown by immunoblotting that bovine cortex membranes contain 120, 135 and 150 kDa species of adenylyl cyclase. However, purification of cortex adenylyl cyclase using forskolin affinity chromatography yields preparations containing only the 120 kDa protein (Pfeuffer *et al*, 1985b; Smigel, 1986). Further, in bovine striatum, most immunoreactivity appears to be 150 kDa form, with little of the 120 kDa form (Mollner & Pfeuffer, 1991); forskolin-purified adenylyl cyclase from striatum is a 150 kDa protein, but the efficiency

of the purification is substantially less than that for the cortex (120 kDa) enzyme (Yoshimasa *et al*, 1988). Thus some adenylyl cyclases may have differing affinities for forskolin, leading to differing abilities to be purified using this matrix.

I had also attempted to use the monoclonal antibody BBC-4 raised by Mollner & Pfeuffer (1988) against bovine brain calmodulin-sensitive adenylyl cyclase to immunoprecipitate adenylyl cyclase protein from  $^{32}\text{P}\text{O}_4$ -labeled S49 cells treated with and without 8-Br-cAMP, in order to assess the phosphorylation of the adenylyl cyclase protein *in vivo*. However, there was no recovery of an immunoprecipitated protein which could be the catalytic unit of adenylyl cyclase (measured by iodination of eluted protein). Further, I was also unable to demonstrate a significant level of immunoreactive protein in cell membranes by immunoblotting with this monoclonal antibody. The cloning of an S49 cell adenylyl cyclase reported here should allow the production of more-specific antisera which can then be used to determine the phosphorylation state of the protein.

I have devised another method which should have great potential for purifying adenylyl cyclase, as well as other G protein-regulated effectors. Affinity chromatography using the immobilized  $\alpha$ -subunits of individual G proteins should allow the rapid purification of other proteins to which the activated  $\alpha$ -subunit binds. Pang & Sternweis (1989; 1990) have developed an affinity matrix from immobilized  $\beta\gamma$ -subunits of G proteins, and used this matrix to rapidly purify  $\alpha$ -subunits in an active state from membrane detergent extracts. This purification relies on the ability of inactive  $\alpha$ -subunits to associate with  $\beta\gamma$ -subunits in a heterotrimeric complex. Membrane detergent extracts are mixed with the  $\beta\gamma$ -matrix under activating conditions (10 mM NaF), and association of the  $\alpha$  and  $\beta\gamma$  subunits is promoted by deactivation (addition of EDTA). the matrix is

washed, and  $\alpha$ -subunits are eluted with NaF. Variations in the activation and elution conditions favors purification of distinct  $\alpha$ -subunits (Pang & Sternweis, 1990). This tight but reversible binding of the  $\alpha$  subunits to  $\beta\gamma$  allows the extensive washing required for single-step purification, despite the very hydrophobic nature of the  $\beta\gamma$ -complex.

The biological specificity of the  $\alpha$ -subunits for effectors can be used to similar advantage to purify the effectors themselves. The inactive (GDP-bound)  $\alpha$ -subunits do not bind nor activate effector, while the activated  $\alpha$ -subunits do. The free  $\alpha_s$  protein is sufficient for activation of adenylyl cyclase; the  $\beta\gamma$ -subunits are not required (Northup *et al*, 1983a; 1983b; Iyengar *et al*, 1988). This basic method has been used indirectly and on a small-scale already: Spiegel and coworkers have utilized an antiserum specific for the C-terminus of  $G_s\alpha$  to co-immunoprecipitate adenylyl cyclase activity following activation of the  $G_s$  (Simonds *et al*, 1989; Morris *et al*, 1990). Rather than using an antiserum to immobilize the  $\alpha_s$ , the protein would be covalently linked to an agarose matrix for affinity chromatography. Free  $\alpha$ -subunits, either purified or (better) prepared by expression of the recombinant  $\alpha$ -subunit cDNA, can be linked through their C-terminus without affecting the ability to be activated and bind effectors. A selection for functional recombinant  $\alpha_s$  protein could easily be made using  $\beta\gamma$ -subunit affinity chromatography. Various linkages would have to be tried to determine which has the least effect on the functional properties of the G protein.

Adenylyl cyclase purification using this procedure has the potential for allowing a single-step purification protocol. Membrane extracts prepared under basal (native  $G_s$  inactive) conditions are added to the  $\alpha$ -subunit matrix and NaF is added to 10 mM to activate the matrix  $\alpha_s$ . Adenylyl cyclase binds to the activated G protein, and the matrix

is washed in the continued presence of NaF. Elution is accomplished by washing the matrix without the NaF activator. This type of purification is ideal for labile trace proteins such as adenylyl cyclase, since it is rapid, allows extensive washing, and requires no harsh elution conditions. Other effectors could be purified using the appropriate  $\alpha$ -subunit matrix, and for  $\alpha$ -subunits whose effectors are not known, one could even purify the unknown effector and assay for various candidate activities. Unfortunately, I have not had time to explore the use of this technique for purifying adenylyl cyclase or other effectors, but I believe this technique can be developed into a valuable tool for G protein-coupled signal transduction research.

In order to further address the role of phosphorylation of adenylyl cyclase in desensitization, I took advantage of the recent cloning of a bovine brain cDNA encoding calmodulin-sensitive adenylyl cyclase (Krupinski *et al*, 1989) to screen for the liver calmodulin-insensitive adenylyl cyclase. Instead of a liver-specific adenylyl cyclase form, however, I have identified two distinct enzymes (types 5 and 6) in the rat liver which are members of a novel subfamily of mammalian adenylyl cyclases (distinct from cloned types 1-4). Both the type 5 and 6 enzymes are widely distributed in rat tissues, and would appear to be the enzyme forms responsible for adenylyl cyclase activity measured in most tissues. Both adenylyl cyclases share a protein kinase A site not found in other cloned mammalian adenylyl cyclases, which may be the site responsible for negative feedback regulation by protein kinase A phosphorylation. The type 5 enzyme has also been cloned from the mouse S49 lymphoma cell line, where adenylyl cyclase activity is also regulated by protein kinase A. Specific antipeptide antisera being prepared against each of these adenylyl cyclase forms may be useful in identifying the native proteins, and

perhaps allowing their immunoprecipitation. This would allow direct determination as to whether these proteins are phosphorylated by protein kinase A in the cell in response to hormones.

The recent (and ongoing) discovery of the diversity of mammalian adenylyl cyclases raises several questions about how adenylyl cyclases are regulated. Until very recently, there were commonly assumed to be two distinct mammalian  $G_s$ -regulated adenylyl cyclases: calmodulin-sensitive enzyme in the brain, and calmodulin-insensitive enzyme everywhere else (Neer, 1978b). The originally cloned type 1 enzyme represents the calmodulin-sensitive form (Smigel, 1986; Krupinski *et al*, 1989; Tang *et al*, 1991). The type 3 enzyme appears limited to the olfactory system; a specialized form of  $G_s$  also occurs in the olfactory system (Jones & Reed, 1989; Bakalyar & Reed, 1990). With the type 5 and 6 adenylyl cyclases reported here, there are at least two distinct subfamilies of somatic calmodulin-insensitive adenylyl cyclases, the type 2/4 family (Feinstein *et al*, 1991; Gao & Gilman, 1991) and the type 5/6 family. An additional partial adenylyl cyclase sequence isolated from human brain may represent an additional subfamily (Parma *et al*, 1991).

Of these cloned adenylyl cyclases, only the types 4, 5 and 6 have been shown to have broad mRNA tissue distributions. The distribution and relative levels of any of the various adenylyl cyclase proteins has yet to be ascertained. Specific anti-peptide antisera against the type 5 and 6 adenylyl cyclases, and against a domain common to all cloned mammalian adenylyl cyclases, are being raised and characterized. These antisera may be useful in mapping the distribution of these adenylyl cyclase enzymes in various tissues.

Functional studies have revealed an unexpected stimulation of the type 2 and 4

subfamily of adenylyl cyclases by  $\beta\gamma$ -subunits of G proteins in the presence of  $G_s$  stimulation (Tang & Gilman, 1991; Gao & Gilman, 1991; Federman *et al*, 1992). This stimulation does not appear to occur in the absence of  $G_s$  stimulation, and therefore represents a novel, conditional form of crosstalk between the adenylyl cyclase system and other G protein-mediated ( $\beta\gamma$ -producing) signaling pathways. The type 5 adenylyl cyclase is not stimulated by  $\beta\gamma$  in this manner, and type 6 probably will not either since it is most similar to type 5. Further regulatory features of the adenylyl cyclases are also predicted to segregate according to the subfamilies predicted in Chapter 6.

The type 4 adenylyl cyclase has no predicted phosphorylation consensus sites for protein kinase A. Type 5 has one predicted site, and type 6 two. One of these sites is conserved between the two related enzymes. Since the type 5 enzyme is in liver and in S49 cells, this form may be negatively regulated by protein kinase A phosphorylation. Type 6 will probably be regulated in the same manner. Type 4 is probably insensitive to protein kinase A phosphorylation. Therefore, the ratio of type 4 and types 5 and 6 in a tissue may determine the effectiveness of negative feedback through protein kinase A. Similarly, the amount of stimulation by  $\beta\gamma$  subunits will also be determined by the ratio of the type 2/4 adenylyl cyclases to the type 5/6 enzymes. Other differences among the adenylyl cyclases (calmodulin-sensitivity, inhibition by  $G_i$ , "P"-site inhibition, efficacy of forskolin stimulation and ability to bind to forskolin-agarose affinity matrix, protein kinase C effects) will also vary depending on the properties of the various individual adenylyl cyclase forms present.

It had been known previously that adenylyl cyclase in several systems can be directly regulated by protein kinase C phosphorylation, which results in increased

hormonal stimulation adenylyl cyclase activity (Bell *et al*, 1984; Johnson *et al*, 1986; Yoshimasa *et al*, 1987). Such phosphorylation could result from hormonal activation phospholipase C enzymes in the cell, which releases diacylglycerol and (indirectly)  $\text{Ca}^{2+}$  which activate the activity of protein kinase C. Rat hepatocytes treated with the phorbol ester TPA or diacylglycerol analogs appear to become desensitized, presumably due to phosphorylation of the glucagon receptor, and additional glucagon treatment induces no further changes in activity (Heyworth *et al*, 1984b; 1985; Murphy *et al*, 1989). Treatment of chick hepatocytes with TPA did not block glucagon-stimulated adenylyl cyclase activity, but at high concentrations did slightly elevate NaF stimulation. In this system, there is no conclusive evidence for protein kinase C-mediated crosstalk due to phosphorylation of either the glucagon receptor or adenylyl cyclase. With expressed individual adenylyl cyclases, the effect of protein kinase C-mediated phosphorylation of each enzyme can now be assessed.

Thus the unveiling of the diversity of adenylyl cyclases which serve as the effector for receptors coupled to  $G_s$  leads to a great potential complexity in signal transduction. With probes for individual adenylyl cyclases, it will now be possible to explore further aspects of adenylyl cyclase regulation, such as control of expression levels. Receptors have been shown to influence their own synthesis through modulation of mRNA levels and gene transcription (Collins *et al*, 1989). Whether adenylyl cyclase protein or mRNA levels are also regulated by cAMP is completely unknown. Colin *et al* (1991) have examined the regulation of adenylyl cyclase mRNAs in the rat brain following lithium treatment, however, and found that type 1 and type 2 adenylyl cyclase mRNAs were elevated compared to untreated controls. If cells can also modulate the levels of adenylyl

cyclase protein in response to hormones, they may also be capable of altering the ratio of distinct adenylyl cyclases they express to match changing needs with altered signaling properties.

## REFERENCES

- Amatruda III, T. T., Steele, D. A., Slepak, V. Z., and Simon, M. I. (1991)  $G\alpha_{16}$ , a G protein  $\alpha$  subunit specifically expressed in hematopoietic cells. Proc. Natl. Acad. Sci. USA **88**: 5587-5591.
- Anholt, R. R. H., and Rivers, A. M. (1990) Olfactory transduction: cross-talk between second messenger systems. Biochemistry **29**: 4049-4054.
- Attramadal, H., Eikvar, L., and Hansson, V. (1988) Mechanisms of glucagon-induced homologous and heterologous desensitization of adenylate cyclase in membranes and whole Sertoli cells of the rat. Endocrinology **123**: 1060-1068.
- Bakalyar, H. A., and Reed, R. R. (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science **250**: 1403-1406.
- Barber, R. (1988) Forskolin binding to intact S49 cells. Second Messengers and Phosphoproteins **12**: 59-71.
- Bayad, J., Sabolovic, N., Bagrel, D., Magdalou, J., and Siest, G. (1991) Influence of the isolation method on the stability of differentiated phenotype in cultured rat hepatocytes. J. Pharmacol. Methods **25**: 85-94.
- Bell, G. I., Santerre, R. F., and Mullenbach, G. T. (1983) Hamster preproglucagon contains the sequence of glucagon and two related peptides. Nature **302**: 716-718.
- Bell, J. D., Buxton, I. L. O., and Brunton, L. L. (1985) Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. Putative effect of C kinase on  $\alpha_s$ -GTP-catalytic subunit interaction. J. Biol. Chem. **260**: 2625-2628.
- Bender, J. L., and Neer, E. J. (1983) Properties of the adenylate cyclase catalytic unit from caudate nucleus. J. Biol. Chem. **258**: 2432-2439.
- Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989)  $\beta$ -Adrenergic receptor kinase: primary structure delineates a multigene family. Science **246**: 235-240.
- Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1987a) Functional desensitization of the isolated  $\beta$ -adrenergic receptor by the  $\beta$ -adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proc. Natl. Acad. Sci. USA **84**: 8879-8882.
- Benovic, J. L., Mayor Jr, F., Somers, R. L., Caron, M. G., and Lefkowitz, R. J. (1986a) Light-dependent phosphorylation of rhodopsin by  $\beta$ -adrenergic receptor kinase. Nature **321**: 869-872.
- Benovic, J. L., Mayor Jr, F., Staniszewski, C., Lefkowitz, R. J., and Caron, M. G. (1987b) Purification and characterization of the  $\beta$ -adrenergic receptor kinase. J. Biol. Chem. **262**: 9026-9032.
- Benovic, J. L., Onorato, J. J., Arriza, J. L., Stone, W. C., Lohse, M., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Caron, M. G., and Lefkowitz, R. J. (1991) Cloning, expression, and chromosomal localization of  $\beta$ -adrenergic receptor kinase 2. A new member of the receptor kinase family. J. Biol. Chem. **266**: 14939-14946.

Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1985) Phosphorylation of the mammalian  $\beta$ -adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. J. Biol. Chem. **260**: 7094-7101.

Benovic J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986b)  $\beta$ -Adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. Proc. Natl. Acad. Sci. USA **83**: 2797-2801.

Berry, M. N., and Friend, D. S. (1969) High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine-structure study. J. Cell Biology **43**: 506-520.

Bigay, J., Deterre, P., Pfister, C., and Chabre, M. (1985) Fluoroaluminates activate transducin-GDP by mimicking the  $\gamma$ -phosphate of GTP in its binding site. FEBS Lett. **191**: 181-185.

Bilofsky, H. S., and Burks, C. (1988) The GenBank (R) genetic sequence data bank. Nucleic Acids Res. **16**: 1861-1864.

Birnbaum, M. J., Haspel, H. C., and Rosen, O. M. (1986) Cloning and characterization of a cDNA encoding the rat brain glucose-transporter protein. Proc. Natl. Acad. Sci. USA **83**: 5784-5788.

Bissell, D. M., and Guzelian, P. S. (1980) Phenotypic stability of adult rat hepatocytes in primary culture. Ann. NY Acad. Sci. USA **349**: 85-98.

Bitonti, A. J., Moss, J., Hjelmeland, L., and Vaughn, M. (1982) Resolution and activity of adenylate cyclase components in a zwitterionic cholate derivative [3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate]. Biochemistry **21**: 3650-3653.

Bokoch, G. M., Katada, T., Northup, J. K., Ui, M., and Gilman, A. G. (1984) Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. J. Biol. Chem. **259**: 3560-3567.

Bookbinder, L. H., Moy, G. W., and Vacquier, V. D. (1991) *In vitro* phosphorylation of sea urchin sperm adenylate cyclase by cyclic adenosine monophosphate-dependent protein kinase. Mol. Reprod. Dev. **28**: 150-157.

Bourne, H. R., Coffino, P., and Tomkins, G. M. (1975) Selection of a variant lymphoma cell deficient in adenylate cyclase. Science **187**: 750-752.

Boyer, J. L., Waldo, G. L., Evans, T., Northup, J. K., Downes, C. P., and Harden, T. K. (1989) Modification of  $\text{AlF}_4^-$  and receptor-stimulated phospholipase C activity by G-protein  $\beta\gamma$  subunits. J. Biol. Chem. **264**: 13917-13922.

Brandt, D. R., Asano, T., Pedersen, S. E., and Ross, E. M. (1983) Reconstitution of catecholamine-stimulated guanosinetriphosphatase activity. Biochemistry **22**: 4357-4362.

Brandt, D. R., and Ross, E. M. (1985) GTPase activity of the stimulatory GTP-binding regulatory protein of adenylate cyclase,  $G_s$ . J. Biol. Chem. **260**: 266-272.

Brandt, D. R., and Ross, E. M. (1986) Catecholamine-stimulated GTPase cycle. Multiple sites of regulation by  $\beta$ -adrenergic receptor and  $Mg^{2+}$  studied in reconstituted phospholipid receptor- $G_i$  vesicles. J. Biol. Chem. **261**: 1656-1664.

Braun, T., and Dods, R. F. (1975) Development of a  $Mn^{2+}$ -sensitive, "soluble" adenylate cyclase in rat testis. Proc. Natl. Acad. Sci. USA **72**: 1097-1101.

Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A., and Nirenberg, M. (1986) Human cDNA clones for four species of  $G\alpha$ , signal transduction protein. Proc. Natl. Acad. Sci. USA **83**: 8893-8897.

Brostrom, C. O., Brostrom, M. A., and Wolff, D. J. (1977) Calcium-dependent adenylate cyclase from rat cerebral cortex. Reversible activation by sodium fluoride. J. Biol. Chem. **252**: 5677-5685.

Brostrom, C. O., Huang, Y., Breckenridge, B. McL., and Wolff, D. J. (1975) Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase. Proc. Natl. Acad. Sci. USA **72**: 64-68.

Burnham, C. E., Hawelu-Johnson, C. L., Frank, B. M., and Lynch, K. R. (1987) Molecular cloning of rat renin cDNA and its gene. Proc. Natl. Acad. Sci. USA **84**: 5605-5609.

Bushfield, M., Griffiths, S. L., Murphy, G. J., Pyne, N. J., Knowler, J. T., Milligan, G., Parker, P. J., Mollner, S., and Houslay, M. D. (1990) Diabetes-induced alterations in the expression, functioning and phosphorylation state of the inhibitory guanine nucleotide regulatory protein  $G_{i2}$  in hepatocytes. Biochem. J. **271**: 365-372.

Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G., and Sefton, B. M. (1987) Myristoylated  $\alpha$  subunits of guanine nucleotide-binding regulatory proteins. Proc. Natl. Acad. Sci. USA **84**: 7493-7497.

Carlson, K. E., Brass, L. F., and Manning, D. R. (1989) Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than  $G_i$  in human platelets. J. Biol. Chem. **264**: 13298-13305.

Carty, D. J., and Iyengar, R. (1990) A 43 kDa form of the GTP-binding protein  $G_{43}$  in human erythrocytes. FEBS Lett. **262**: 101-103.

Casey, P. J., Fong, H. K. W., Simon, M. I., and Gilman, A. G. (1990)  $G_z$ , a guanine nucleotide-binding protein with unique biochemical properties. J. Biol. Chem. **265**: 2383-2390.

Cassel, D., and Pfeuffer, T. (1978) Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide binding protein of the adenylate cyclase system. Proc. Natl. Acad. Sci. USA **75**: 2669-2673.

Cassel, D., and Selinger, Z. (1976) Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. Biochem. Biophys. Acta **452**: 538-551.

Cassel, D., and Selinger, Z. (1977) Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. Proc. Natl. Acad. Sci. USA **74**: 3307-3311.

Cassel, D., and Selinger, Z. (1978) Mechanism of adenylate cyclase activation through the  $\beta$ -adrenergic receptor: catecholamine-induced displacement of bound GDP by GTP. Proc. Natl. Acad. Sci. USA **75**: 4155-4159.

Cassill, J. A., Whitney, M., Joazeiro, C. A. P., Becker, A., and Zuker, C. S. (1991) Isolation of *Drosophila* genes encoding G protein-coupled receptor kinases. Proc. Natl. Acad. Sci. USA **88**: 11067-11070.

Cerione, R. A., Regan, J. W., Nakata, H., Codina, J., Benovic, J. L., Gierschik, P., Somers, R. L., Spiegel, A. M., Birnbaumer, L., Lefkowitz, R. J., and Caron, M. G. (1986) Functional reconstitution of the  $\alpha_2$ -adrenergic receptor with guanine regulatory proteins in phospholipid vesicles. J. Biol. Chem. **261**: 3901-3909.

Chang, F.-H., and Bourne, H. R. (1987) Dexamethasone increases adenylyl cyclase activity and expression of the  $\alpha$ -subunit of  $G_i$  in GH<sub>3</sub> cells. Endocrinology **121**: 1711-1715.

Chen, C.-J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. Cell **47**: 381-389.

Cheung, A. H., Dixon, R. A. F., Hill, W. S., Sigal, I. S., and Strader, C. D. (1990) Separation of the structural requirements for agonist-promoted activation and sequestration of the  $\beta$ -adrenergic receptor. Mol. Pharmacol. **37**: 775-779.

Cheung, A. H., Sigal, I. S., Dixon, R. A. F., and Strader, C. D. (1989) Agonist-promoted sequestration of the  $\beta_2$ -adrenergic receptor requires regions involved in functional coupling with  $G_s$ . Mol. Pharmacol. **34**: 132-138.

Cheung, W. Y., Bradham, L. D., Lynch, T. J., Lin, Y. M., and Tallant, E. A. (1975) Protein activator of cyclic 3':5'-nucleotide phosphodiesterase of bovine or rat brain also activates its adenylyl cyclase. Biochem. Biophys. Res. Commun. **66**: 1055-1062.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acids from sources enriched in ribonuclease. Biochemistry **18**: 5294-5299.

Citri, Y., and Schramm, M. (1980) Resolution, reconstitution and kinetics of the primary action of a hormone receptor. Nature **287**: 297-300.

Clark, R. B. (1986) Desensitization of hormonal stimuli coupled to regulation of cyclic AMP levels. In: *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*. Vol. 20 (Greengard, P. and Robison, G. A., eds) Raven Press, New York pp. 151-209.

Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader, C. D. (1989) Identification of a specific site required for rapid heterologous desensitization of the  $\beta$ -adrenergic receptor by cAMP-dependent protein kinase. Mol. Pharmacol. **36**: 343-348.

Clark, R. B., Friedman, J., Johnson, J. A., and Kunkel, M. W. (1987)  $\beta$ -Adrenergic receptor desensitization of wild-type but not *cyc*<sup>-</sup> lymphoma cells unmasked by submillimolar Mg<sup>2+</sup>. FASEB J. **1**: 289-297.

Clark, R. B., Goka, T. J., Green, D. A., Barber, R., and Butcher, R. W. (1982) Differences in the forskolin activation of adenylyl cyclases in wild-type and variant lymphoma cells. Mol. Pharmacol. **22**: 609-613.

Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J., and Johnson, J. A. (1988) Activation of cAMP-dependent protein kinase is required for heterologous desensitization of adenylyl cyclase in S49 wild-type lymphoma cells. Proc. Natl. Acad. Sci. USA **85**: 1442-1446.

Codina, J., Hildebrandt, J. D., Birnbaumer, L., and Sekura, R. D. (1984a) Effects of guanine nucleotides and Mg on human erythrocyte  $N_i$  and  $N_o$ , the regulatory components of adenylyl cyclase. J. Biol. Chem. **259**: 11408-11418.

Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, C. R., Iyengar, R., and Birnbaumer, L. (1984b)  $N_o$  and  $N_i$ , the stimulatory and inhibitory regulatory components of adenylyl cyclase. Purification of the human erythrocyte proteins without the use of activating ligands. J. Biol. Chem. **259**: 5871-5886.

Coffino, P., Bourne, H. R., and Tomkins, G. M. (1975) J. Cell Physiol. **85**: 603-610.

Colin, S. F., Chang, H.-C., Mollner, S., Pfeuffer, T., Reed, R. R., Duman, R. S., and Nestler, E. J. (1991) Chronic lithium regulates the expression of adenylate cyclase and  $G_i$ -protein in rat cerebral cortex. Proc. Natl. Acad. Sci. USA **88**: 10634-10637.

Collins, S., Bouvier, M., Bolanowski, M. A., Caron, M. G., and Lefkowitz, R. J. (1989) cAMP stimulates transcription of the  $\beta_2$ -adrenergic receptor gene in response to short-term agonist exposure. Proc. Natl. Acad. Sci. USA **86**: 4853-4857.

Coussen, F., Guermah, M., d'Alayer, J., Monneron, A., Haiech, J., and Cavadore, J.-C. (1986) Evidence for two distinct adenylate cyclase catalysts in rat brain. FEBS Lett. **206**: 213-217.

Coussen, F., Haiech, J., D'Alayer, J., and Monneron, A. (1985) Identification of the catalytic subunit of brain adenylate cyclase: a calmodulin binding protein of 135 kDa. Proc. Natl. Acad. Sci. USA **82**: 6736-6740.

Crouch, M. F., Winegar, D. A., and Lapetina, E. G. (1989) Epinephrine induces changes in the subcellular distribution of the inhibitory GTP-binding protein  $G_{i2}$  and a 38-kDa phosphorylated protein in the human platelet. Proc. Natl. Acad. Sci. USA **86**: 1776-1780.

Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R., and Edelman, G. M. (1987) Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science **236**: 799-806.

de Diego, J. G., Alvarez, E., and Blasquez, E. (1987) Characterization of glucagon receptors in Golgi fractions of fetal rat liver. FEBS Lett. **222**: 256-260.

DeRubertis, F. R., and Craven, P. (1976) Reduced sensitivity of the hepatic adenylate cyclase-cyclic AMP system to glucagon during sustained hormonal stimulation. J. Clin. Invest. **57**: 435-443.

Dixon, R. A. F., Sigal, I. J., Rands, E., Register, R. B., Candelore, M. R., Blake, A. D., and Strader, C. D. (1987) Ligand binding to the  $\beta$ -adrenergic receptor involves its rhodopsin-like core. Nature **326**: 73-77.

Dohlman, H. G., Caron, M. G., and Lefkowitz, R. J. (1987) A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry **26**: 2657-2664.

- Dohlman, H. G., Caron, M. G., Strader, C. D., Amlaiky, N., and Lefkowitz, R. J. (1988) Identification and sequence of a binding site peptide of the  $\beta_2$ -adrenergic receptor. Biochemistry 27: 1813-1817.
- Doss, R. C., Kramercy, N. R., Harden, T. K., and Perkins, J. P. (1985) Effects of tunicamycin on the expression of  $\beta$ -adrenergic receptors in human astrocytoma cells during growth and recovery from agonist-induced down-regulation. Mol. Pharmacol. 27: 507-516.
- Doss, R. C., Perkins, J. P., and Harden, T. K. (1981) Recovery of  $\beta$ -adrenergic receptors following long term exposure of astrocytoma cells to catecholamine. Role of protein synthesis. J. Biol. Chem. 256: 12281-12286.
- Drucker, D. J., and Asa, S. (1988) Glucagon gene expression in vertebrate brain. J. Biol. Chem. 263: 13475-13478.
- Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987) Protein serine/threonine kinases. Ann. Rev. Biochemistry 56: 567-613.
- Eliot, L. S., Dudai, Y., Kandel, E. R., and Abrams, T. W. (1989)  $\text{Ca}^{2+}$ /calmodulin sensitivity may be common to all forms of neural adenylate cyclase. Proc. Natl. Acad. Sci. 86: 9564-9568.
- Falloon, J., Malech, H., Milligan, G., Unson, C., Kahn, R., Goldsmith, P., and Spiegel, A. (1986) Detection of the major pertussis toxin substrate of human leukocytes with antisera raised against synthetic peptides. FEBS Lett. 209: 352-356.
- Farah, A. E. (1983) Glucagon and the circulation. Pharmacol. Rev. 35: 181-217.
- Feder, D., Im, M.-J., Klein, H. W., Hekman, M., Holzhöfer, A., Dees, C., Levitzki, A., Helmreich, E. J. M., and Pfeuffer, T. (1986) Reconstitution of  $\beta_1$ -adrenoceptor-dependent adenylate cyclase from purified components. EMBO J. 5: 1509-1514.
- Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R., and Bourne, H. R. (1992) Hormonal stimulation of adenylyl cyclase through  $G_i$ -protein  $\beta\gamma$  subunits. Nature 356: 159-161.
- Feinstein, P. G., Schrader, K. A., Bakalyar, H. A., Tang, W.-J., Krupinski, J., Gilman, A. G., and Reed, R. R. (1991) Molecular cloning and characterization of a  $\text{Ca}^{2+}$ /calmodulin-insensitive adenylyl cyclase from rat brain. Proc. Natl. Acad. Sci. USA 88: 10173-10177.
- Flockhart, D. A., and Corbin, J. D. (1984) Preparation of the catalytic subunit of cAMP-dependent protein kinase. In: *Brain Receptor Methodologies, Part A*. (Marangos, P. J., ed.) Academic Press, Orlando pp. 209-215.
- Florio, V. A., and Ross, E. M. (1983) Regulation of the catalytic component of adenylate cyclase. Potentiative interaction of stimulatory ligands and 2',5'-dideoxyadenosine. Mol. Pharmacol. 24: 195-202.
- Fong, H. K. W., Yoshimoto, K. K., Eversole-Cire, P., and Simon, M. I. (1988) Identification of a GTP-binding protein  $\alpha$  subunit that lacks an apparent ADP-ribosylation site for pertussis toxin. Proc. Natl. Acad. Sci. USA 85: 3066-3070.

Friedman, K. D., Rosen, N. L., Newman, P. J., and Montgomery, R. R. (1990) Screening of  $\lambda$ gt11 libraries. In: *PCR Protocols*. (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) Academic Press, San Diego pp.253-258.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**: 8998-9002.

Fukada, Y., Ohguro, H., Saito, T., Yoshizawa, T., and Akino, T. (1989)  $\beta\gamma$ -Subunit of bovine brain transducin composed of two components with distinctive  $\gamma$ -subunits. J. Biol. Chem. **264**: 5937-5943.

Fung, B. K.-K., Hurley, J. B., and Stryer, L. (1981) Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. USA **78**: 152-156.

Fung, B. K.-K., and Stryer, L. (1980) Photolyzed rhodopsin catalyzes the exchange of GTP for GDP in retinal rod outer segment membranes. Proc. Natl. Acad. Sci. USA **77**: 2500-2504.

Gao, B., Gilman, A. G., and Robishaw, J. D. (1987) A second form of the  $\beta$  subunit of signal-transducing G proteins. Proc. Natl. Acad. Sci. USA **84**: 6122-6125.

Gao, B., and Gilman, A. G. (1991) Cloning and expression of a widely distributed (type IV) adenylyl cyclase. Proc. Natl. Acad. Sci. USA **88**: 10178-10182.

Gao, B., Mumby, S., and Gilman, A. G. (1988) The G protein  $\beta_2$  complementary DNA encodes the  $\beta_{35}$  subunit. J. Biol. Chem. **162**: 17254-17527.

Garrity, M. J., Andreassen, T. J., Storm, D. R., and Robertson, R. P. (1983) Prostaglandin  $E_1$ -induced heterologous desensitization of hepatic adenylyl cyclase. Consequences on the guanyl nucleotide regulatory complex. J. Biol. Chem. **258**: 8692-8697.

Gautam, N., Baetscher, M., Aebersold, R., and Simon, M. I. (1989) A G protein  $\gamma$  subunit shares homology with *ras* proteins. Science **244**: 971-974.

Gautam, N., Northup, J., Tamir, H., and Simon, M. I. (1990) G protein diversity is increased by associations with a variety of  $\gamma$  subunits. Proc. Natl. Acad. Sci. **87**: 7973-7977.

Gibbons, I. R., Asai, D. J., Ching, N. S., Dolecki, G. J., Mocz, G., Phillipson, C. A., Ren, H., Tang, W.-J. Y., and Gibbons, B. H. (1991) A PCR procedure to determine the sequence of large polypeptides by rapid walking through a cDNA library. Proc. Natl. Acad. Sci. USA **88**: 8563-8567.

Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. Ann. Rev. Biochemistry **56**: 615-649.

Green, A., Milligan, G., and Dobias, S. B. (1992)  $G_i$  down-regulation as a mechanism for heterologous desensitization in adipocytes. J. Biol. Chem. **267**: 3223-3229.

Green, J. P. (1990) Pharmacological receptors: the need for a compendium of classification, nomenclature and structure. TIPS **11**: 13-16.

Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T. M., Sutcliffe, J. G., and Lerner, R. A. (1982) Immunogenic structure of the influenza virus hemagglutinin. Cell **28**: 477-487.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) The preparation of  $^{131}\text{I}$ -labelled human growth hormone of high specific radioactivity. Biochem. J. **89**: 114-123.

Gros, P., Croop, J., and Houseman, D. (1986) Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell **47**: 371-380.

Gundersen, R. E., and Devreotes, P. N. (1990) In vivo receptor-mediated phosphorylation of a G protein in *Dictyostelium*. Science **248**: 591-593.

Gurr, J. A., and Ruh, T. A. (1980) Desensitization of primary cultures of adult rat liver parenchymal cells to stimulation of adenosine 3',5'-monophosphate production by glucagon and epinephrine. Endocrinology **107**: 1309-1319.

Haga, K., and Haga, T. (1990) Dual regulation by G proteins of agonist-dependent phosphorylation of muscarinic acetylcholine receptors. FEBS Lett. **268**: 43-47.

Haga, K., and Haga, T. (1992) Activation by G protein  $\beta\gamma$  subunits of agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin. J. Biol. Chem. **267**: 2222-2227.

Haga, T., and Haga, K. (1989) Agonist-dependent phosphorylation of cerebral and atrial muscarinic receptors: blockade of the phosphorylation by GTP-binding regulatory proteins and its reversal by guanine nucleotides. Biomed. Res. **10**: 293-299.

Haga, T., Ross, E. M., Anderson, H. J., and Gilman, A. G. (1977) Adenylate cyclase permanently uncoupled from hormone receptors in a novel variant of S49 mouse lymphoma cells. Proc. Natl. Acad. Sci. USA **74**: 2016-2020.

Halliday, K. R. (1984) Regional homology in GTP-binding proto-oncogene products and elongation factors. J. Cyclic Nucl. Protein Phosphor. Res. **9**: 435-448.

Haraguchi, K., and Rodbell, M. (1990) Isoproterenol stimulates shift of G proteins from plasma membrane to pinocytotic vesicles in rat adipocytes: a possible means of signal dissemination. Proc. Natl. Acad. Sci. USA **87**: 1208-1212.

Harden, T. K. (1983) Agonist-induced desensitization of the  $\beta$ -adrenergic receptor-linked adenylate cyclase. Pharmacol. Rev. **35**: 5-32.

Harden, T. K., Su, Y.-F., and Perkins, J. P. (1979) Catecholamine-induced desensitization involves an uncoupling of  $\beta$ -adrenergic receptors and adenylate cyclase. J. Cyclic Nucleotide Res. **5**: 99-106.

Harris, B. A., Robishaw, J. D., Mumby, S. M., and Gilman, A. G. (1985) Molecular cloning of complementary DNA for the  $\alpha$ -subunit of the G protein that stimulates adenylate cyclase. Science **229**: 1274-1277.

Harrison, J. K., Hewlett, G. H. K., and Gnegy, M. E. (1989) Regulation of calmodulin-sensitive adenylate cyclase by the stimulatory G-protein,  $G_s$ . J. Biol. Chem. **264**: 15880-15885.

Hasegawa, S., Terazono, K., Nata, K., Takada, T., Yamamoto, H., and Okamoto, H. (1990) Nucleotide sequence determination of chicken glucagon precursor cDNA. Chicken preproglucagon does not contain glucagon-like peptide II. FEBS Lett. **264**: 117-120.

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) Phosphorylation sites on two domains of the  $\beta_2$ -adrenergic receptor are involved in distinct pathways of receptor desensitization. J. Biol. Chem. **264**: 12657-12665.

Hermouet, S., Meredino, J. J., Gutkind, J. S., and Spiegel, A. M. (1991) Activating and inactivating mutations of the  $\alpha$  subunit of  $G_{12}$  protein have opposite effects on proliferation of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA **88**: 10455-10459.

Hescheler, J., Rosenthal, W., Trautwein, W., and Schultz (1987) The GTP-binding protein  $G_o$  regulates neuronal calcium channels. Nature **325**: 445-447.

Heyworth, C. M., Hanski, E., and Houslay, M. D. (1984a) Islet-activating protein blocks glucagon desensitization in intact hepatocytes. Biochem. J. **222**: 189-194.

Heyworth, C. M., and Houslay, M. D. (1983) Challenge of hepatocytes by glucagon triggers a rapid modulation of adenylate cyclase activity in isolated membranes. Biochem. J. **214**: 93-98.

Heyworth, C. M., Whetton, A. D., Kinsella, A. R., and Houslay, M. D. (1984b) The phorbol ester, TPA inhibits glucagon-stimulated adenylate cyclase activity. FEBS Lett. **170**: 38-42.

Heyworth, C. M., Wilson, S. P., Gawler, D. J., and Houslay M. D. (1985) The phorbol ester TPA prevents the expression of both glucagon desensitization and the glucagon-mediated block of insulin stimulation of the peripheral plasma membrane cyclic AMP phosphodiesterase in rat hepatocytes. FEBS Lett. **187**: 196-200

Hide, M., Ali, H., Price, S. R., Moss, J., and Beaven, M. A. (1991) GTP-binding protein  $G_{\alpha_z}$ : its down-regulation by dexamethasone and its credentials as a mediator of antigen-induced responses in RBL-2H3 cells. Mol. Pharmacol. **40**: 473-479.

Hildebrandt, J. D., Codina, J., Risinger, R., and Birnbaumer, L. (1984) Identification of a  $\gamma$  subunit associated with the adenylyl cyclase regulatory proteins  $N_s$  and  $N_i$ . J. Biol. Chem. **259**: 2039-2042.

Hildebrandt, J. D., Sekura, R. D., Codina, J., Iyengar, R., Manclark, C. R., and Birnbaumer, L. (1983) Stimulation and inhibition of adenylyl cyclases mediated by distinct regulatory proteins. Nature **302**: 706-709.

Hirn, M., Pierres, M., Deagostini-Bazin, H., Hirsch, M., and Gordis, C. (1981) Monoclonal antibody against cell surface glycoprotein of neurons. Brain Res. **214**: 433-439.

Homcy, C., Wrenn, S., and Haber, E. (1978) Affinity purification of cardiac adenylate cyclase: dependence on prior hydrophobic resolution. Proc. Natl. Acad. Sci. USA **75**: 59-63.

Huang, J., Eng, J., and Yalow, R. S. (1987) Chicken glucagon: sequence and potency in receptor assay. Horm. Metabol. Res. **19**: 542-544.

Hurley, J. T., Fong, H. K. W., Teplow, D. B., Dreyer, W. J., and Simon, M. I. (1984a) Isolation and characterization of a cDNA clone for the  $\gamma$  subunit of bovine retinal transducin. Proc. Natl. Acad. Sci. USA **81**: 6948-6952.

Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D., and Gilman A. G. (1984b) Homologies between signal transducing G proteins and *ras* gene products. Science **226**: 860-862.

Imoto, Y., Yatani, A., Reeves, J. P., Codina, J., Birnbaumer, L., and Brown, A. M. (1988)  $\alpha$ -Subunit of  $G_i$  activates cardiac calcium channels in lipid bilayers. Am. J. Physiol. **255**: H722-H728.

Innes, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.) (1990) *PCR Protocols. A guide to methods and applications*. Academic Press, San Diego.

Insel, P. A., Bourne, H. R., Coffino, P., and Tomkins, G. M. (1975) Cyclic AMP-dependent protein kinase: pivotal role in regulation of enzyme induction and growth. Science **190**: 896-898.

Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K., and Nagata, S. (1991) Molecular cloning and expression of a cDNA encoding the secretin receptor. EMBO J. **10**: 1635-1641.

Iwanij, V., and Vincent, A. C. (1990) Characterization of the glucagon receptor and its functional domains using monoclonal antibodies. J. Biol. Chem. **265**: 21302-21308.

Iyengar, R., and Birnbaumer, L. (1981) Hysteretic activation of adenylyl cyclases. I. Effect of Mg ion on the rate of activation by guanine nucleotides and fluoride. J. Biol. Chem. **256**: 11036-11041.

Iyengar, R., and Birnbaumer, L. (1982) Hormone receptor modulates the regulatory component of adenylyl cyclase by reducing its requirement for  $Mg^{2+}$  and enhancing its extent of activation by guanine nucleotides. Proc. Natl. Acad. Sci. USA **79**: 5179-5183.

Iyengar, R., and Birnbaumer, L. (1987) Signal transduction by G-proteins. ISI Atlas Sci: Pharmacol. **1**: 213-221.

Iyengar, R., and Herberg, J. T. (1984) Structural analysis of the hepatic glucagon receptor. Identification of a guanine nucleotide-sensitive hormone-binding region. J. Biol. Chem. **259**: 5222-5229.

Iyengar, R., Mintz, P. W., Swartz, T. L., and Birnbaumer, L. (1981b) Divalent cation-induced desensitization of glucagon-stimulable adenylyl cyclase in rat liver plasma membrane. GTP-dependent stimulation by glucagon. J. Biol. Chem. **255**: 11875-11882.

Iyengar, R., Rich, K. A., Herberg, J. T., Grenet, D., Mumby, S., and Codina, J. (1987) Identification of a new GTP-binding protein. A  $M_r=43,000$  substrate for pertussis toxin. J. Biol. Chem. **262**: 9239-9245.

Iyengar, R., Rich, K. A., Herberg, J. T., Premont, R. T., and Codina, J. (1988) Glucagon receptor-mediated activation of  $G_i$  is accompanied by subunit dissociation. J. Biol. Chem. **263**: 15348-15353.

Jakobs, K. H., Aktories, K., and Shultz, G. (1983) A nucleotide regulatory site for somatostatin inhibition of adenylyl cyclase in S49 lymphoma cells. Nature **303**: 177-178.

Jena, B. P., and Abramowitz, J. (1989) Human chorionic gonadotropin-induced heterologous desensitization of rabbit luteal adenylyl cyclase is associated with altered receptor and G-protein function. Endocrinology **124**: 1932-1941.

Johnson, G. L., MacAndrew Jr, V. I., and Pilch, P. F. (1981) Identification of the glucagon receptor in rat liver membranes by photoaffinity crosslinking. Proc. Natl. Acad. Sci. USA **78**: 875-878.

Johnson, J. A., Goka, T. J., and Clark, R. B. (1986) Phorbol ester-induced augmentation and inhibition of epinephrine-stimulated adenylate cyclase in S49 lymphoma cells. J. Cyclic Nucleotide Prot. Phosphor. Res. **11**: 199-215.

Johnson, R. A. (1982) An approach to the identification of adenosine's inhibitory site on adenylate cyclase. FEBS Lett. **140**: 80-84.

Johnson, R. A., Saeur, W., and Jakobs, K. H. (1979) Effects of prostaglandin E<sub>1</sub> and adenosine on metal and metal-ATP kinetics of platelet adenylate cyclase. J. Biol. Chem. **254**: 1094-1101.

Jones, D. T., Masters, S. B., Bourne, H. R., and Reed, R. R. (1990a) Biochemical characterization of three stimulatory GTP-binding proteins. The large and small forms of G<sub>s</sub> and the olfactory-specific G-protein G<sub>olf</sub>. J. Biol. Chem. **265**: 2671-2676.

Jones, D. T., and Reed, R. R. (1987) Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. J. Biol. Chem. **262**: 14241-14249.

Jones, D. T., and Reed, R. R. (1989) G<sub>olf</sub>, an olfactory neuron specific-G protein involved in odorant signal transduction. Science **244**: 790-795.

Jones, T. L. Z., Simonds, W. F., Merendino, J. J. Jr., Brann, M. R., and Spiegel, A. M. (1990b) Myristoylation of an inhibitory GTP-binding protein  $\alpha$  subunit is essential for its membrane attachment. Proc. Natl. Acad. Sci. USA **87**: 568-572.

Kassis, S., and Fishman, P. H. (1982) Different mechanisms of desensitization of adenylate cyclase by isoproterenol and prostaglandin E<sub>1</sub> in human fibroblasts. Role of regulator components in desensitization. J. Biol. Chem. **257**: 5312-5318.

Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S., and Jakobs, K. H. (1985) Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. Eur. J. Biochem. **151**: 431-437.

Kataoka, T., Broek, D., and Wigler, M. (1985) DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. Cell **43**: 493-505.

Keenan, A. K., Cooney, D., Holzhofer, A., Dees, C., and Hekman, M. (1987) Unimpaired coupling of phosphorylated, desensitized  $\beta$ -adrenoceptor to G<sub>s</sub> in a reconstituted system. FEBS Lett. **217**: 287-291.

Kim, U.-H., Kim, J. W., and Rhee, S. G. (1989) Phosphorylation of phospholipase C- $\gamma$  by cAMP-dependent protein kinase. J. Biol. Chem. **264**: 20167-20170.

Kirchick, H. J., Iyengar, R., and Birnbaumer, L. (1983) Human chorionic gonadotropin-induced heterologous desensitization of adenylyl cyclase from highly luteinized rat ovaries: attenuation of regulatory N component activity. Endocrinology **113**: 1638-1646.

Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G., and Lefkowitz, R. J. (1988) Chimeric  $\alpha_2$ - $\beta_2$ -adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. Science **240**: 1310-1316.

Koesling, D., Böhme, E., and Schultz, G. (1991) Guanylyl cyclases, a growing family of signal-transducing enzymes. FASEB J. **5**: 2785-2791.

Kozasa, T., Itoh, H., Tsukamoto, T., and Kaziro, Y. (1988) Isolation and characterization of the human  $G_i$ - $\alpha$  gene. Proc. Natl. Acad. Sci. USA **85**: 2081-2085.

Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., and Gilman, A. G. (1989) Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science **244**: 1558-1564.

Krupp, M., and Lane, M. D. (1981) On the mechanism of ligand-induced down regulation of insulin receptor level in the liver cell. J. Biol. Chem. **256**: 1689-1694.

Kunkel, M. W., Friedman, J., Shenolikar, S., and Clark, R. B. (1989) Cell-free heterologous desensitization of adenylyl cyclase in S49 lymphoma cell membranes mediated by cAMP-dependent protein kinase. FASEB J. **3**: 2067-2074.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

Laurenza, A., Sutkowski, E. McH., and Seamon, K. B. (1989) Forskolin: a specific stimulator of adenylyl cyclase or a diterpine with multiple sites of action? TIPS **10**: 442-447.

Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N., Clapham, D., and Peralta, E. (1990) Distinct sequence elements control the specificity of G protein activation by muscarinic acetylcholine receptor subtypes. EMBO J. **9**: 4381-4390.

Lee, E., Taussig, R., and Gilman, A. G. (1992) The G226A mutant of  $G_{s\alpha}$  highlights the requirement for dissociation of G protein subunits. J. Biol. Chem. **267**: 1212-1218.

Levin, L. R., Han, P.-L., Hwang, P. M., Feinstein, P. G., Davis, R. L., and Reed, R. R. (1992) The *Drosophila* learning and memory gene *rutabaga* encodes a  $Ca^{2+}$ /calmodulin-responsive adenylyl cyclase. Cell **68**: 479-489.

Levine, M. A., Smallwood, P. M., Moen, P. T., Helman, L. J., and Ahn, T. G. (1990) Molecular cloning of  $\beta_3$  subunit, a third form of the G protein  $\beta$ -subunit polypeptide. Proc. Natl. Acad. Sci. USA **87**: 2329-2333.

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.-J., Dumont, J. E., and Vassart, G. (1989) Selective amplification and cloning of four new members of the G protein-coupled receptor family. Science **244**: 569-572.

Liggett, S. B., Bouvier, M., Hausdorff, W. P., O'Dowd, B., Caron, M. G., and Lefkowitz, R. J. (1989) Altered patterns of agonist-stimulated cAMP accumulation in cells expressing mutant  $\beta_2$ -adrenergic receptors lacking phosphorylation sites. Mol. Pharmacol. **36**: 641-646.

Linder, M. E., Ewald, D. A., Miller, R. J., and Gilman, A. G. (1990) Purification and characterization of  $G_o\alpha$  and three types of  $G_{i\alpha}$  after expression in *Escherichia coli*. J. Biol. Chem. **265**: 8243-8251.

Lipkin, V. M., Khramtsov, N. V., Andreeva, S. G., Moshnyakov, M. V., Petukhova, G. V., Rakitina, T. V., Feshchenko, E. A., Ishchenko, K. A., Mirzoeva, S. F., Chernova, M. N., and Dranytsyna, S. M. (1989b) Calmodulin-independent bovine brain adenylate cyclase. Amino acid sequence and nucleotide sequence of the corresponding cDNA. FEBS Lett. **254**: 69-73.

Lipkin, V. M., Mirzoeva, S. F., Dranitsina, S. M., Moshnyakov, M. V., Petrov, V. M., Chernova, M. N., Surina, E. A., Obukhov, A. N., Levina, N. B., Khramtsov, N. V., Andreeva, S. G., Rakitina, T. V., Feshchenko, E. A., and Ovchinnikov, Yu. A. (1989a) Catalytic component of calmodulin-independent adenylate cyclase from bovine brain. Isolation and determination of partial amino acid sequence (Russian). Bioorg. Khim. **15**: 5-17.

Lipson, K. E., Kolhatkar, A. A., Cherskey, B. D., and Donner, D. B. (1986) Characterization of glucagon receptors in Golgi fractions of rat liver: evidence for receptors that are uncoupled from adenylate cyclase. Biochemistry **25**: 2612-2620.

Livingstone, M. S. (1985) Genetic dissection of *Drosophila* adenylate cyclase. Proc. Natl. Acad. Sci. USA **82**: 5992-5996.

Livingstone, M. S., Sziber, P. P., and Quinn, W. G. (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of *rutabaga*, a *Drosophila* learning mutant. Cell **37**: 205-215.

Loh, E. (1991) Anchored PCR: amplification with single-sided specificity. Methods: A Companion to Methods in Enzymology **2**: 11-19.

Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990)  $\beta$ -Arrestin: a protein that regulates  $\beta$ -adrenergic receptor function. Science **248**: 1547-1550.

Londos, C., and Preston, M. S. (1977) Regulation by glucagon and divalent cations of inhibition of hepatic adenylate cyclase by adenosine. J. Biol. Chem. **252**: 5951-5956.

Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J., and Rodbell, M. (1974) 5'-Guanylyl-imidodiphosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. Proc. Natl. Acad. Sci. USA **71**: 3087-3090.

Longabaugh, J. P., Didsbury, J., Spiegel, A., and Stiles, G. L. (1989) Modification of the rat adipocyte  $A_1$  adenosine receptor-adenylate cyclase system during chronic exposure to an  $A_1$  adenosine receptor agonist: alterations in the quantity of  $G_s$ - $\alpha$  and  $G_i$ - $\alpha$  are not associated with changes in their mRNAs. Mol. Pharmacol. **36**: 681-688.

Lopez, L. C., Frazier, M. L., Su, C.-J., Kumar, A., and Saunders, G. F. (1983) Mammalian pancreatic preproglucagon contains three glucagon-related peptides. Proc. Natl. Acad. Sci. USA **80**: 5485-5489.

Lorenz, W., Inglese, J., Palczewski, K., Onorato, J. J., Caron, M. G., and Lefkowitz, R. J. (1991) The receptor kinase family: primary structure of rhodopsin kinase reveals similarities to the  $\beta$ -adrenergic receptor kinase. Proc. Natl. Acad. Sci. USA **88**: 8715-8719.

Lounsbury, K. M., Casey, P. J., Brass, L. F., and Manning, D. R. (1991) Phosphorylation of  $G_z$  in human platelets. Selectivity and site of modification. J. Biol. Chem. **266**: 22051-22056.

Lowndes, J. M., Gupta, S. K., Osawa, S., and Johnson, G. L. (1991) GTPase-deficient  $G\alpha_{12}$  oncogene *gip2* inhibits adenylate cyclase and attenuates receptor-stimulated phospholipase  $A_2$  activity. J. Biol. Chem. **266**: 14193-14197.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**: 265-275.

Luetje, C. W., and Nathanson, N. M. (1988) Chronic membrane depolarization regulates the level of the guanine nucleotide binding protein  $G_i$ - $\alpha$  in cultured neuronal cells. J. Neurochem. **50**: 1775-1782.

Mahan, L. C., Koachman, A. M., and Insel, P. C. (1985) Genetic analysis of  $\beta$ -adrenergic receptor internalization and down-regulation. Proc. Natl. Acad. Sci. USA **82**: 129-133.

Manning, D. R., Fraser, B. A., Kahn, R. A., and Gilman, A. G. (1984) ADP-ribosylation of transducin by islet-activating protein. Identification of asparagine as the site of ADP-ribosylation. J. Biol. Chem. **259**: 749-756.

Marchetti, C., Premont, R. T., and Brown, A. M. (1988) A whole-cell and single-channel study of the voltage dependent outward potassium current in avian hepatocytes. J. Gen. Physiol. **91**: 255-274.

Masson, P., Lenzen, G., Jacquemin, J. M., and Danchin, A. (1986) Yeast adenylate cyclase catalytic domain is carboxy terminal. Curr. Genet. **10**: 343-352.

Masters, S. B., Sullivan, K. A., Miller, R. T., Beiderman, B., Lopez, N. G., Ramachandran, J., and Bourne, H. R. (1988) Carboxyl terminal domain of  $G_i$ - $\alpha$  specifies coupling of receptors to stimulation of adenylyl cyclase. Science **241**: 448-451.

Matsui, H., Lefkowitz, R. J., Caron, M. G., and Regan, J. W. (1989) Localization of the fourth membrane spanning domain as a ligand binding site in the human platelet  $\alpha_2$ -adrenergic receptor. Biochemistry **28**: 4125-4130.

Matsuoka, M., Itoh, H., Kosaza, T., and Kaziro, Y. (1988) Sequence analysis of cDNA and genomic DNA for a putative pertussis toxin-insensitive guanine nucleotide-binding regulatory protein  $\alpha$  subunit. Proc. Natl. Acad. Sci. USA **85**: 5384-5388.

Mattera, R., Graziano, M. P., Yatani, A., Zhou, Z., Graf, R., Codina, J., Birnbaumer, L., Gilman, A. G., and Brown, A. M. (1989a) Splice variants of the  $\alpha$  subunit of the G protein  $G_i$  activate both adenylyl cyclase and calcium channels. Science **243**: 804-807.

May, D. C., Ross, E. M., Gilman, A. G., and Smigel, M. D. (1985) Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins. J. Biol. Chem. **260**: 15829-15833.

Milligan, G., and Green, A. (1991) Agonist control of G-protein levels. TIPS **12**: 207-209.

Minocherhomjee, A. M., Selfe, S., Flowers, N. J., and Storm, D. R. (1987) Direct interaction between the catalytic subunit of the calmodulin-sensitive adenylate cyclase from bovine brain with  $^{125}$ I-labeled wheat germ agglutinin and  $^{125}$ I-labeled calmodulin. Biochemistry **26**: 4444-4448.

Mochly-Rosen, D., Chang, F.-H., Cheever, L., Kim, M., Diamond, I., and Gordon, A. S. (1988) Chronic ethanol causes heterologous desensitization of receptors by reducing  $\alpha$ , messenger RNA. Nature **333**: 848-850.

Mollner, S., and Pfeuffer, T. (1988) Two different adenylyl cyclases in brain distinguished by monoclonal antibodies. Eur. J. Biochem. **171**: 265-271.

Mollner, S., and Pfeuffer, T. (1991) Characteristics and use of monoclonal antibodies to various forms of adenylyl cyclase. In: *Adenylyl Cyclase, G Proteins, and Guanylyl Cyclase, Methods in Enzymology* volume 195 (R. A. Johnson and J. D. Corbin, eds.) Academic Press, San Diego pp. 121-129.

Mollner, S., Simmoteit, R., Palm, D., and Pfeuffer, T. (1991) Monoclonal antibodies against various forms of the adenylyl cyclase catalytic subunit and associated proteins. Eur. J. Biochem. **195**: 281-286.

Monneron, A., d'Alayer, J., and Coussen, F. (1987) Purification of the catalytic subunit of adenylate cyclase in vertebrates: state of the art in 1987. Biochimie **69**: 263-269.

Moriarty, T. M., Gillo, B., Carty, D. J., Premont, R. T., Landau, E. M., and Iyengar, R. (1988)  $\beta\gamma$  Subunits of GTP-binding proteins inhibit muscarinic receptor stimulation of phospholipase C. Proc. Natl. Acad. Sci. USA **85**: 8865-8869.

Moriarty, T. M., Padrell, E., Carty, D. J., Omri, G., Landau, E. M., and Iyengar, R. (1990)  $G_o$  protein as signal transducer in the pertussis toxin-sensitive phosphatidylinositol pathway. Nature **343**: 79-82.

Morris, D. I., McHugh-Sutkowski, E., Moos, M. Jr., Simonds, W. F., Spiegel, A. M., and Seamon, K. B. (1990) Immunoprecipitation of adenylate cyclase with an antibody to a carboxy-terminal peptide from  $G_{\alpha}$ . Biochemistry **29**: 9079-9084.

Morris, D. I., Speicher, L. A., Ruoho, A. E., Tew, K. D., and Seamon, K. B. (1991) Interaction of forskolin with the P-glycoprotein multidrug transporter. Biochemistry **30**: 8371-8379.

Moss, J., Tsai, S.-C., Adamik, R., Chen, H.-C., and Stanley, S. J. (1988) Purification and characterization of ADP-ribosylarginine hydrolase from turkey erythrocytes. Biochemistry **27**: 5819-5823.

Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E., and Lodish, H. F. (1985) Sequence and structure of a human glucose transporter. Science **229**: 941-945.

Mumby, S. M., Heukeroth, R. O., Gordon, J. L., and Gilman, A. G. (1990) G-protein  $\alpha$ -subunit expression, myristoylation, and membrane attachment in COS cells. Proc. Natl. Acad. Sci. USA **87**: 728-732.

Mumby, S. M., Kahn, R. A., Manning, D. R., and Gilman, A. G. (1986) Antisera of designed specificity for subunits of guanine nucleotide-binding regulatory proteins. Proc. Natl. Acad. Sci. USA **83**: 265-269.

Murayama, T., and Ui, M. (1984)  $[^3H]GDP$  release from rat and hamster adipocyte membranes independently linked to receptors involved in activation or inhibition of adenylate cyclase. Differential susceptibility to two bacterial toxins. J. Biol. Chem. **259**: 761-769.

Murphy, G. J., Gawler, D. J., Milligan, G., Wakelam, M. J. O., Pyne, N. J., and Houslay, M. D. (1989) Glucagon desensitization of adenylate cyclase and stimulation of inositol phospholipid metabolism does not involve the inhibitory guanine nucleotide regulatory protein  $G_i$ , which is inactivated upon challenge of hepatocytes with glucagon. Biochem. J. **259**: 191-197.

Murphy, G. J., and Houslay, M. D. (1988) Resensitization of hepatocyte glucagon-stimulated adenylate cyclase can be inhibited when cyclic AMP phosphodiesterase inhibitors are used to elevate intracellular cyclic AMP concentrations to supraphysiological values. Biochem. J. **249**: 543-547.

Murphy, G. J., Hruby, V. J., Trevidi, D., Wakelam, M. J. O., and Houslay, M. D. (1987) The rapid desensitization of glucagon-stimulated adenylate cyclase is a cAMP-independent process that can be mimicked by hormones which stimulate inositol phospholipid metabolism. Biochem. J. **243**: 39-46.

Neer, E. J. (1978a) Physical and functional properties of adenylate cyclase from mature rat testis. J. Biol. Chem. **253**: 5808-5812.

Neer, E. J. (1978b) Multiple forms of adenylate cyclase. In: *Advances in Cyclic Nucleotide Research* (W. J. George and L. J. Ignarro, eds.) Raven Press, New York **9**: 69-83.

Neer, E. J., Lok, J. M., and Wolf, L. G. (1984) Purification and properties of the inhibitory guanine regulatory unit of brain adenylate cyclase. J. Biol. Chem. **259**: 14222-14229.

Nelson, C. A., and Seamon, K. B. (1986) Binding of [<sup>3</sup>H]forskolin to human platelet membranes. Regulation by guanyl-5'-yl imidodiphosphate, NaF, and prostaglandins E<sub>1</sub> and D<sub>2</sub>. J. Biol. Chem. **261**: 13469-13473.

Nelson, C. A., and Seamon, K. B. (1988) Binding of [<sup>3</sup>H]forskolin to solubilized preparations of adenylate cyclase. Life Sci. **42**: 1375-1383.

Neville, D. R. (1968) Isolation of an organ-specific protein antigen from cell-surface membranes of rat liver. Biochem. Biophys. Acta **154**: 540-552.

Newlands, C., and Houslay, M. D. (1991) Treatment of intact hepatocytes with synthetic diacyl glycerols mimics the ability of glucagon to cause the desensitization of adenylate cyclase. FEBS Lett. **289**: 129-132.

Noda, C., Shinjyo, F., Tomomura, A., Kato, S., Nakamura, T., and Ichihara, A. (1984) Mechanism of heterologous desensitization of the adenylate cyclase system by glucagon in primary cultures of adult rat hepatocytes. J. Biol. Chem. **259**: 7747-7754.

Northup, J. K., Smigel, M. D., and Gilman, A. G. (1982) The guanine nucleotide activating site of the regulatory component of adenylate cyclase: identification by ligand binding. J. Biol. Chem. **257**: 11416-11423.

Northup, J. K., Smigel, M. D., Sternweis, P. C., and Gilman, A. G. (1983a) The subunits of the stimulatory regulatory component of adenylyl cyclase. Resolution of the activated 45,000-dalton ( $\alpha$ ) subunit. J. Biol. Chem. **258**: 11369-11376.

Northup, J. K., Sternweis, P. C., and Gilman, A. G. (1983b) The subunits of the stimulatory regulatory component of adenylyl cyclase. Resolution, activity, and properties of the 35,000-dalton ( $\beta$ ) subunit. J. Biol. Chem. **258**: 11361-11368.

O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. (1988) Site-directed mutagenesis of the cytoplasmic domains of the human  $\beta_2$ -adrenergic receptor. Localization of regions involved in G protein-receptor coupling. J. Biol. Chem. **263**: 15985-15992.

Padrell, E., Carty, D. J., Moriarty, T. M., Hildebrandt, J. D., Landau, E. M., and Iyengar, R. (1991) Two forms of the bovine brain  $G_o$  that stimulate the inositol trisphosphate-mediated  $Cl^-$  currents in *Xenopus* oocytes. Distinct guanine nucleotide binding properties. J. Biol. Chem. **266**: 9771-9777.

Padrell, E., Herberg, J. T., Monsatirsky, B., Floyd, G., Premont, R. T., and Iyengar, R. (1987) The hepatic glucagon receptor: a comparative study of the regulatory and structural properties. Endocrinology **120**: 2316-2325.

Pang, I.-H., and Sternweis, P. C. (1989) Isolation of the  $\alpha$  subunits of GTP-binding regulatory proteins by affinity chromatography with immobilized  $\beta\gamma$  subunits. Proc. Natl. Acad. Sci. USA **86**: 7814-7818.

Pang, I.-H., and Sternweis, P. C. (1990) Purification of unique  $\alpha$  subunits of GTP-binding regulatory proteins (G proteins) by affinity chromatography with immobilized  $\beta\gamma$  subunits. J. Biol. Chem. **265**: 18707-18712.

Parma, J., Stengel, D., Gannage, M.-H., Poyard, M., Barouki, R., and Hanoune, J. (1991) Sequence of a human brain adenylyl cyclase partial cDNA. Evidence for a consensus cyclase specific domain. Biochem. Biophys. Res. Comm. **179**: 455-462.

Parsons, W. J., and Stiles, G. L. (1987) Heterologous desensitization of the inhibitory  $A_1$  adenosine receptor-adenylate cyclase system in rat adipocytes. Regulation of both  $N_s$  and  $N_q$ . J. Biol. Chem. **262**: 841-847.

Pearson, W. R., and Lipman, D. J. (1988) Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**: 2444-2448.

Pedersen, S. E., and Ross, E. M. (1982) Functional reconstitution of  $\beta$ -adrenergic receptors and the stimulatory GTP-binding protein of adenylyl cyclase. Proc. Natl. Acad. Sci. USA **79**: 7228-7232.

Peterson, J. E., Larew, J. S.-A., and Graves, D. J. (1990) Purification and partial characterization of arginine-specific ADP-ribosyltransferase from skeletal muscle microsomal membranes. J. Biol. Chem. **265**: 17062-17069.

Pfeuffer, E., Dreher, R.-M., Metzger, H., and Pfeuffer, T. (1985a) Catalytic unit of adenylyl cyclase: purification and identification by affinity crosslinking. Proc. Natl. Acad. Sci. USA **82**: 3086-3090.

Pfeuffer, E., Mollner, S., Lancet, D., and Pfeuffer, T. (1989) Olfactory adenylyl cyclase. Identification and purification of a novel enzyme form. J. Biol. Chem. **264**: 18803-18807.

Pfeuffer, E., Mollner, S., and Pfeuffer, T. (1985b) Adenylyl cyclase from bovine brain cortex: purification and characterization of the catalytic subunit. EMBO J. **4**: 3675-3679.

Pfeuffer, T. (1991) Synthesis of forskolin-agarose affinity matrices. In: *Adenylyl Cyclase, G Proteins, and Guanylyl Cyclase, Methods in Enzymology* volume 195 (R. A. Johnson and J. D. Corbin, eds.) Academic Press, San Diego pp. 44-51.

Pfeuffer, T., and Metzger, H. (1982) 7-O-Hemisuccinyl-deacetyl forskolin-Sepharose: a novel affinity support for purification of adenylyl cyclase. FEBS Lett. **146**: 369-375.

Premont, R. T. (1991) A bovine brain cDNA purported to encode calmodulin-insensitive adenylyl cyclase has extensive identity with neural cell adhesion molecules (N-CAMs). *FEBS Lett.* **295**: 230-231.

Premont, R. T., Buku, A., and Iyengar, R. (1989) The  $G\alpha_z$  gene product in human erythrocytes. Identification as a 41-kiloDalton protein. *J. Biol. Chem.* **264**: 14960-14964.

Premont, R. T., Chen, J., Ponnappalli, M., and Iyengar, R. (1992) Cloning of two members of a new subfamily of hormone-stimulated adenylyl cyclases. *Proc. Natl. Acad. Sci. USA* (manuscript submitted).

Premont, R. T., and Iyengar, R. (1987) Glucagon receptor: structure and function. In: *Peptide Hormone Receptors*. (Kalimi, M. Y., and Hubbard, J. R., eds) Walter de Gruyter & Co., Berlin New York pp. 129-177.

Premont, R. T., and Iyengar, R. (1988) Glucagon-induced desensitization of adenylyl cyclase in primary cultures of chick hepatocytes. Evidence for multiple pathways. *J. Biol. Chem.* **263**: 16087-16095.

Premont, R. T., and Iyengar, R. (1989) Heterologous desensitization of the liver adenylyl cyclase: analysis of the role of G proteins. *Endocrinology* **125**: 1151-1160.

Premont, R. T., and Iyengar, R. (1990a) Adenylyl cyclase and its regulation by  $G_i$ . In: *G Proteins*. (Iyengar, R., and Birnbaumer, L., eds) Academic Press, San Diego pp. 157-178.

Premont, R. T., and Iyengar, R. (1990b) Multiple pathways for glucagon-induced heterologous desensitization of liver adenylyl cyclase. In: *Activation and Desensitization of Signal Transducing Pathways*. NATO ASI Series, Vol. H44 (Konijn, T. M., Houslay, M. D., and van Haastert, P. J. M., eds) Springer-Verlag, Berlin pp. 85-97.

Premont, R. T., and Iyengar, R. (1992) Liver adenylyl cyclases: structure and regulation by cAMP-dependent phosphorylation. In: *Adenine Nucleotides in Cellular Energy Transfer and Metabolism*. (Papa, S., Azzi, A., and Trager, J. M., eds.) Birkhäuser Verlag, Basel (IN PRESS).

Pyne, N. J., Murphy, G. J., Milligan, G., and Houslay, M. D. (1989) Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein  $G_i$ . *FEBS Lett.* **243**: 77-82.

Refnes, M., Johansen, E. J., and Christoffersen, T. (1989) Glucagon-induced refractoriness of hepatocyte adenylate cyclase: comparison of homologous and heterologous components and evidence against a role of cAMP. *Pharmacol. Toxicol.* **64**: 397-403.

Reithmann, C., Gierschik, P., Werdan, K., and Jakobs, K. H. (1991) Role of inhibitory G protein  $\alpha$ -subunits in adenylyl cyclase desensitization. *Mol. Cell. Endocrinol.* **82**: C215-C221.

Retrosen, D., Gallin, J. I., Spiegel, A. M., and Malech, H. L. (1988) Subcellular localization of  $G_i$ - $\alpha$  in human neutrophils. *J. Biol. Chem.* **263**: 10958-10964.

Rich, K. A., Codina, J., Floyd, G., Sekura, R., Hildebrandt, J. D., and Iyengar, R. (1984) Glucagon-induced heterologous desensitization of the MDCK cell adenylyl cyclase. *J. Biol. Chem.* **259**: 7893-7901.

Rich, K. A., and Iyengar, R. (1989) Desensitization of the  $\beta$ -adrenergic receptor of the S49 lymphoma cell: mechanism of receptor loss and recovery. J. Mol. Endocrinology 3: 219-227.

Riordan, J. R., Rommens, J. M., Kerem, B.-S. Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245: 1066-1073.

Robishaw, J. D., Kalman, V. K., Moomaw, C. R., and Slaughter, C. A. (1989) Existence of two  $\gamma$  subunits of the G proteins in brain. J. Biol. Chem. 264: 15758-15761.

Robishaw, J. D., Russell, D. W., Harris, B. A., Smigel, M. D., and Gilman, A. G. (1986a) Deduced primary structure of the  $\alpha$  subunit of the GTP-binding stimulatory protein of adenylate cyclase. Proc. Natl. Acad. Sci. USA 83: 1251-1255.

Robishaw, J. D., Smigel, M. D., and Gilman, A. G. (1986b) Molecular basis for two forms of the G protein that stimulates adenylate cyclase. J. Biol. Chem. 261: 9587-9590.

Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. J. (1971a) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. J. Biol. Chem. 246: 1877-1882.

Rodbell, M., Krans, H. M. J., Pohl, S. J., and Birnbaumer, L. (1971c) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. IV. Effects of guanyl nucleotides on binding of  $^{125}$ I-glucagon. J. Biol. Chem. 246: 1872-1876.

Rojas, F. J., Swartz, T. L., Iyengar, R., Garber, A. J., and Birnbaumer, L. (1983) Monoiodoglucagon: synthesis, purification by high pressure liquid chromatography, and characteristics as a receptor probe. Endocrinology 113: 711-719.

Ros, M., Northup, J. K., and Malbon, C. C. (1988) Steady-state levels of G-proteins and  $\beta$ -adrenergic receptors in rat fat cells. Permissive effects of thyroid hormones. J. Biol. Chem. 263: 4362-4368.

Rosenberg, G. B., and Storm, D. R. (1987) Immunological distinction between calmodulin-sensitive and calmodulin-insensitive adenylate cyclases. J. Biol. Chem. 262: 7623-7628.

Ross, E. M., and Gilman, A. G. (1977a) Resolution of some components of adenylate cyclase necessary for catalytic activity. J. Biol. Chem. 252: 6966-6969.

Ross, E. M., and Gilman, A. G. (1977b) Reconstitution of catecholamine-sensitive adenylate cyclase activity: interaction of solubilized components with receptor-replete membranes. Proc. Natl. Acad. Sci. USA 74: 3715-3719.

Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., and Gilman, A. G. (1977) Relationship between the  $\beta$ -adrenergic receptor and adenylate cyclase. Studies of ligand binding and enzyme activity in purified membranes of S49 lymphoma cells. J. Biol. Chem. 252: 5761-5775.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of  $\beta$ -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

Salomon, Y., Lin, M. C., Londos, C., Rendall, M., and Rodbell, M. (1975) The hepatic adenylate cyclase system. I. Evidence for transition states and structural requirements for guanine nucleotide activation. J. Biol. Chem. **250**: 4239-4245.

Salomon, Y., Londos, C., and Rodbell, M. (1974) A highly sensitive adenylate cyclase assay. Anal. Biochem **58**: 541-548.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (eds.) (1989) *Molecular Cloning. A laboratory manual*. Second edition Cold Spring Harbor Laboratory Press.

Santos, A., and Blazquez, E. (1982) Direct evidence of a glucagon-dependent regulation of the concentration of glucagon receptors in the liver. Eur. J. Biochem. **121**: 671-677.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51**: 660-672.

Schmid, A., Renaud, J.-F., and Lazdunski, M. (1985) Short term and long term effects of  $\beta$ -adrenergic effectors and cyclic AMP on nitrendipine-sensitive voltage-dependent  $\text{Ca}^{2+}$  channels of skeletal muscle. J. Biol. Chem. **260**: 13041-14046.

Schramm, M., and Rodbell, M. (1975) A persistent active state of the adenylate cyclase system produced by the combined actions of isoproterenol and guanylyl imidodiphosphate in frog erythrocyte membranes. J. Biol. Chem. **250**: 2232-2237.

Schultz, J. E., Klumpp, S., Benz, R., Schürhoff-Goeters, W. J. Ch., and Schmid, A. (1992) Regulation of adenylyl cyclase from *Paramecium* by an intrinsic potassium conductance. Science **255**: 600-603.

Seamon, K. B., and Daly, J. W. (1981a) Activation of adenylate cyclase by the diterpine forskolin does not require the guanine nucleotide regulatory protein. J. Biol. Chem. **256**: 9799-9801.

Seamon, K. B., and Daly, J. W. (1981b) Forskolin: a unique diterpine activator of cyclic AMP-generating systems. J. Cyclic. Nucl. Res. **7**: 201-224.

Seamon, K. B., Padgett, W., and Daly, J. W. (1981) Forskolin: unique diterpine activator of adenylate cyclase in membranes and intact cells. Proc Natl Acad. Sci. USA **78**: 3363-3367.

Seamon, K. B., Vaillancourt, R., Edwards, M., and Daly, J. W. (1984) Binding of [ $^3\text{H}$ ]forskolin to rat brain membranes. Proc. Natl. Acad. Sci. USA **81**: 5081-5085.

Shchenkova, I. M., and Kuznetsova, L. A. (1984) Comparative assay of biological activity of chicken and mammalian glucagons in experiments *in vitro*. J. Evol. Biochem. Physiol. (Engl. trans.) **20**: 539-542.

Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1988)  $\lambda$ ZAP: a bacteriophage  $\lambda$  expression vector with *in vivo* excision properties. Nucleic Acids Res. **16**: 7583-7600.

Sibley, D. R., Jeffs, R. A., Daniel, K., Nambi, P., and Lefkowitz, R. J. (1986a) Phorbol diester treatment promotes enhanced adenylate cyclase activity in frog erythrocytes. Arch. Biochem. Biophys. **244**: 373-381.

Sibley, D. R., and Lefkowitz, R. J. (1988) Biochemical mechanisms of  $\beta$ -adrenergic receptor regulation. ISI Atlas Sci: Pharmacol. 2: 66-70.

Sibley, D. R., and Lefkowitz, R. J. (1985) Molecular mechanisms of receptor desensitization using the  $\beta$ -adrenergic receptor-coupled adenylate cyclase as a model. Nature 317: 124-129.

Sibley, D. R., Peters, J. R., Nambi, P., Caron, M. G., and Lefkowitz, R. J. (1984b) Desensitization of turkey erythrocyte adenylate cyclase.  $\beta$ -Adrenergic receptor phosphorylation is correlated with attenuation of adenylate cyclase activity. J. Biol. Chem. 259: 9742-9749.

Simmoteit, R., Schulzki, H.-D., Palm, D., Mollner, S., and Pfeuffer, T. (1991) Chemical and functional analysis of components of adenylyl cyclase from human platelets treated with phorbol esters. FEBS Lett. 285: 99-103.

Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Diversity of G proteins in signal transduction. Science 252: 802-808.

Simonds, W. F., Goldsmith, P. F., Woodard, C. J., Unson, C. G., and Spiegel, A. M. (1989) Receptor and effector interactions of  $G_q$ . Functional studies with antibodies to the  $\alpha_q$  carboxyl-terminal decapeptide. FEBS Lett. 249: 189-194.

Smigel, M. D. (1986) Purification of the catalyst of adenylate cyclase. J. Biol. Chem. 261: 1976-1982.

Smrcka, A. V., Helper, J. R., Brown, K. O., and Sternweis, P. C. (1991) Regulation of polyphosphatidylinositol-specific phospholipase C activity by purified  $G_q$ . Science 251: 804-807.

Sreedharan, S. P., Robichon, A., Peterson, K. E., and Goetzl, E. J. (1991) Cloning and expression of the human vasoactive intestinal peptide receptor. Proc. Natl. Acad. Sci. USA 88: 4986-4990.

Staehelin, M., and Hertel, C. (1983) [ $^3$ H]CGP-12177, a  $\beta$ -adrenergic ligand suitable for measuring cell surface receptors. J. Receptor Res. 3: 35-43.

Starling, E. H. (1905) The chemical correlation of the functions of the body. The chemical control of the functions of the body. Lancet 2: 338-341.

Steinberg, R. A., Wetters, T. van D., and Coffino P. (1978) Kinase-negative mutants of S49 mouse lymphoma cells carry a trans-dominant mutation affecting expression of cAMP-dependent protein kinase. Cell 15: 1351-1361.

Stengel, D., Parma, J., Gannagé, M.-H., Roeckel, N., Mattei, M.-G., Barouki, R., and Hanoune, J. (1992) Different chromosomal localization of two adenylyl cyclase genes expressed in human brain. Human Genetics (in press).

Sternweis, P. C. (1986) The purified  $\alpha$  subunits of  $G_o$  and  $G_i$  from bovine brain require  $\beta\gamma$  for association with phospholipid vesicles. J. Biol. Chem. 261: 631-637.

Sternweis, P. C., and Robishaw, J. D. (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. 259: 13806-13813.

Strader, C. D., Sigal, I. S., Blake, A. D., Cheung, A. H., Register, R. B., Rands, E., Zemcik, B. A., Candelore, M. R., and Dixon, R. A. F. (1987) The carboxyl terminus of the hamster  $\beta$ -adrenergic receptor expressed in mouse L cells is not required for receptor sequestration. Cell 49: 855-863.

Strasser, R. H., and Lefkowitz, R. J. (1985) Homologous desensitization of  $\beta$ -adrenergic receptor coupled adenylate cyclase. Resensitization following polyethylene glycol treatment. J. Biol. Chem. 260: 4561-4564.

Strathmann, M. P., and Simon, M. I. (1990) G protein diversity: a distinct class of  $\alpha$  subunits is present in vertebrates and invertebrates. Proc. Natl. Acad. Sci. USA 87: 9113-9117.

Strathmann, M. P., and Simon, M. I. (1991)  $G\alpha_{12}$  and  $G\alpha_{13}$  subunits define a fourth class of G protein  $\alpha$  subunits. Proc. Natl. Acad. Sci. USA 88: 5582-5586.

Strathmann, M. P., Wilkie, T. M., and Simon, M. I. (1989) Diversity of the G-protein family: sequences from five additional  $\alpha$  subunits in the mouse. Proc. Natl. Acad. Sci. USA 86: 7407-7409.

Strosberg, A. D. (1991) Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. Eur. J. Biochem. 196: 1-10.

Strulovici, B., Cerione, R. A., Kilpatrick, B. F., Caron, M. G., and Lefkowitz, R. J. (1984) Direct demonstration of impaired functionality of a purified desensitized  $\beta$ -adrenergic receptor in a reconstituted system. Nature 225: 837-840.

Su, Y.-F., Harden, T. K., and Perkins, J. P. (1979) Isoproterenol-induced desensitization of adenylate cyclase in human astrocytoma cells. J. Biol. Chem. 254: 38-41.

Su, Y.-F., Harden, T. K., and Perkins, J. P. (1980) Catecholamine-specific desensitization of adenylate cyclase: evidence for a multistep process. J. Biol. Chem. 255: 7410-7419.

Sutherland, E. W., and Rall, T. S. (1958) Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J. Biol. Chem. 232: 1077-1091.

Sutherland, E. W., Rall, T. S., and Menon, T. (1962) Adenyl cyclase. I. Distribution, preparation, and properties. J. Biol. Chem. 237: 1220-1227.

Tamir, H., Fawzi, A. B., Tamir, A., Evans, T., and Northup, J. K. (1991) G-protein  $\beta\gamma$  forms: identity of  $\beta$  and diversity of  $\gamma$  subunits. Biochemistry 30: 3929-3936.

Tang, W.-J., and Gilman, A. G. (1991) Type-specific regulation of adenylyl cyclase by G protein  $\beta\gamma$  subunits. Science 254: 1500-1503.

Tang, W.-J., Krupinski, J., and Gilman, A. G. (1991) Expression and characterization of calmodulin-activated (type I) adenylylcyclase. J. Biol. Chem. 266: 8595-8603.

Tanuma, S., Kawashima, K., and Endo, H. (1988) Eukaryotic mono(ADP-ribosyl)transferase that ADP-ribosylates GTP-binding regulatory  $G_i$  protein. J. Biol. Chem. 263: 5485-5489.

Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., Zwergel, E. E., and Lane, M. D. (1977) Lipogenesis and the synthesis and secretion of very low density lipoprotein by avian liver cells in nonproliferating monolayer culture. J. Cell Biol. 73: 332-353.

Taylor, S. J., Smith, J. A., and Exton, J. H. (1990) Purification from bovine liver membranes of a guanine nucleotide-dependent activator of phosphoinositide-specific phospholipase C. Immunologic identification as a novel G-protein  $\alpha$  subunit. J. Biol. Chem. **265**: 17150-17156.

Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton, J. H. (1991) Activation of the  $\beta 1$  isozyme of phospholipase C by  $\alpha$  subunits of the  $G_q$  class of G proteins. Nature **350**: 516-518.

Toutant, M., Aunis, D., Bockaert, J., Homburger, V., and Rouot, B. (1987) Presence of three pertussis toxin substrates and  $G_o\alpha$  immunoreactivity in both plasma and granule membranes of chromaffin cells. FEBS Lett. **215**: 339-344.

Towbin H., Staehelin T., and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**: 4350-4354.

Unger, R. H. (1985) Glucagon physiology and pathophysiology in the light of new advances. Diabetologia **28**: 574-578.

VanDongen, A. M. J., Codina, J., Olate, J., Mattera, R., Joho, R., Birnbaumer, L., and Brown, A. M. (1988) Newly identified brain potassium channels gated by the guanine nucleotide binding protein  $G_o$ . Science **242**: 1433-1437.

Van Dop, C., Tsubokawa, M., Bourne, H. R., and Ramachandran, J. (1984a) Amino acid sequence of retinal transducin at the site of ADP-ribosylation by cholera toxin. J. Biol. Chem. **259**: 696-698.

Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R. D., Manclark, C. R., Stryer, L., and Bourne, H. R. (1984b) ADP-ribosylation of transducin by pertussis toxin blocks the light-stimulated hydrolysis of GTP and cGMP in retinal photoreceptors. J. Biol. Chem. **259**: 23-26.

Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs, J. B. (1988) Cloning of bovine GAP and its association with oncogenic *ras* p21. Nature **335**: 90-93.

Wadzinski, B. E., Shanahan, M. F., Seamon, K. B., and Ruoho, A. E. (1990) Localization of the forskolin photolabelling site within the monosaccharide transporter of human erythrocytes. Biochem. J. **272**: 151-158.

Waldo, G. L., Doss, R. C., Perkins, J. P., and Harden, T. K. (1984) Use of a density shift method to assess  $\beta$ -adrenergic receptor synthesis during recovery from catecholamine-induced down-regulation in human astrocytoma cells. Mol. Pharmacol. **26**: 424-429.

Waldo, G. L., Northup, J. K., Perkins, J. P., and Harden, T. K. (1983) Characterization of an altered membrane form of the  $\beta$ -adrenergic receptor produced during agonist-induced desensitization. J. Biol. Chem. **258**: 13900-13908.

Walseth, T. F., and Johnson, R. A. (1979) The enzymatic preparation of  $[\alpha\text{-}^{32}\text{P}]\text{NTP}$  and cyclic  $[\text{}^{32}\text{P}]\text{AMP}$  and  $[\text{}^{32}\text{P}]\text{GMP}$ . Biochem. Biophys. Acta **562**: 11-31.

Watkins, P. A., Tarlow, D. M., and Lane, M. D. (1977) Mechanism for acute control of fatty acid synthesis by glucagon and 3':5'-cyclic AMP in the liver cell. Proc. Natl. Acad. Sci. USA **74**: 1497-1501.

West, R. E., Moss, J., Vaughn, M., Liu, T., and Liu, T.-Y. (1985) Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. J. Biol. Chem. **260**: 14428-14430.

Westcott, K. R., LaPorte, D. C., and Storm, D. R. (1979) Resolution of adenylate cyclase sensitive and insensitive to  $\text{Ca}^{2+}$  and calcium-dependent regulatory protein (CDR) by CDR-Sepharose affinity chromatography. Proc. Natl. Acad. Sci. USA **76**: 204-208.

Wilden, U., Hall, S. W., and Kuhn, H. (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc. Natl. Acad. Sci. USA **83**: 1174-1178.

Wong, S. K.-F., Slaughter, C., Ruoho, A. E., and Ross, E. M. (1988) The catecholamine binding site of the  $\beta$ -adrenergic receptor is formed by juxtaposed membrane-spanning domains. J. Biol. Chem. **263**: 7925-7928.

Wong, Y. H., Conklin, B. R., and Bourne, H. R. (1992)  $G_z$ -mediated inhibition of cyclic AMP accumulation. Science **255**: 339-342.

Wong, Y. H., Federman, A., Pace, A. M., Zachary, I., Evans, T., Pouyssegur, J., and bourne, H. R. (1991) Mutant  $\alpha$  subunits of  $G_{12}$  inhibit cyclic AMP accumulation. Nature **351**: 63-65.

Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**: 197-203.

Wu, D., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) Activation of phospholipase C by the  $\alpha$  subunits of the  $G_q$  and  $G_{11}$  proteins in transfected COS-7 cells. J. Biol. Chem. **267**: 1811-1817.

Xia, Z., Refsdal, C. D., Merchant, K. M., Dorsa, D. M., and Storm, D. R. (1991) Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: expression in areas associated with learning and memory. Neuron **6**: 431-443.

Yamashita, A., Kurokawa, T., Une, Y., and Ishibashi, S. (1989) Reduction in the stability of the  $G_s$ -catalytic unit complex of adenylate cyclase in isoproterenol-induced heterologous desensitization. Eur. J. Pharmacology **159**: 247-256.

Yamawaki-Kataoka, Y., Tamaoki, T., Choe, H.-R., Tanaka, H., and Kataoka, T. (1989) Adenylate cyclases in yeast: a comparison of the genes from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **86**: 5693-5697.

Yatani, A., Codina, J., Brown, A. M., and Birnbaumer, L. (1987a) Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein  $G_k$ . Science **235**: 207-211.

Yatani, A., Codina, J., Imoto, Y., Reeves, J. P., Birnbaumer, L., and Brown, A. M. (1987b) A G protein directly regulates mammalian cardiac calcium channels. Science **238**: 1288-1292.

Yatani, A., Mattera, R., Codina, J., Graf, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A. M., and Birnbaumer, L. (1989) The G protein-gated atrial  $\text{K}^+$  channel is stimulated by three distinct  $G_i$   $\alpha$ -subunits. Nature **336**: 680-682.

Yeager, R. E., Heideman, W., Rosenberg, G. B., and Storm, D. R. (1985) Purification of the calmodulin-sensitive adenylate cyclase from bovine cerebral cortex. Biochemistry **24**: 3776-3783.

Yoshimasa, T., Bouvier, M., Benovic, J. L., Amlaiky, N., Lefkowitz, R. J., and Caron, M. G. (1988) Regulation of the adenylate cyclase signaling pathway: potential role for the phosphorylation of the catalytic unit by protein kinase A and protein kinase C. In: *Molecular Biology of Brain and Endocrine Peptidergic Systems*. (McKerns, K. W., and Chretien, M., eds.) Plenum Publishing, New York pp. 123-139.

Yoshimasa, T., Sibley, D. R., Bouvier, M., Lefkowitz, R. J., and Caron, M. G. (1987) Cross-talk between cellular signaling pathways suggested by phorbol ester-induced adenylate cyclase phosphorylation. Nature 327: 67-70.

Zaitseva, F. B., Petrov, V. M., Elistratova, E. Y., and Lipkin, V. M. (1988) Purification and characterization of catalytic unit of calmodulin-sensitive adenylate cyclase from bovine brain cortex (Russian). Biologicheskie Membrany 5: 1125-1134.

Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschik, P., and Spiegel, A. M. (1986) Multisite phosphorylation of the  $\alpha$  subunit of transducin by the insulin receptor kinase and protein kinase C. Proc. Natl. Acad. Sci. USA 83: 9294-9297.