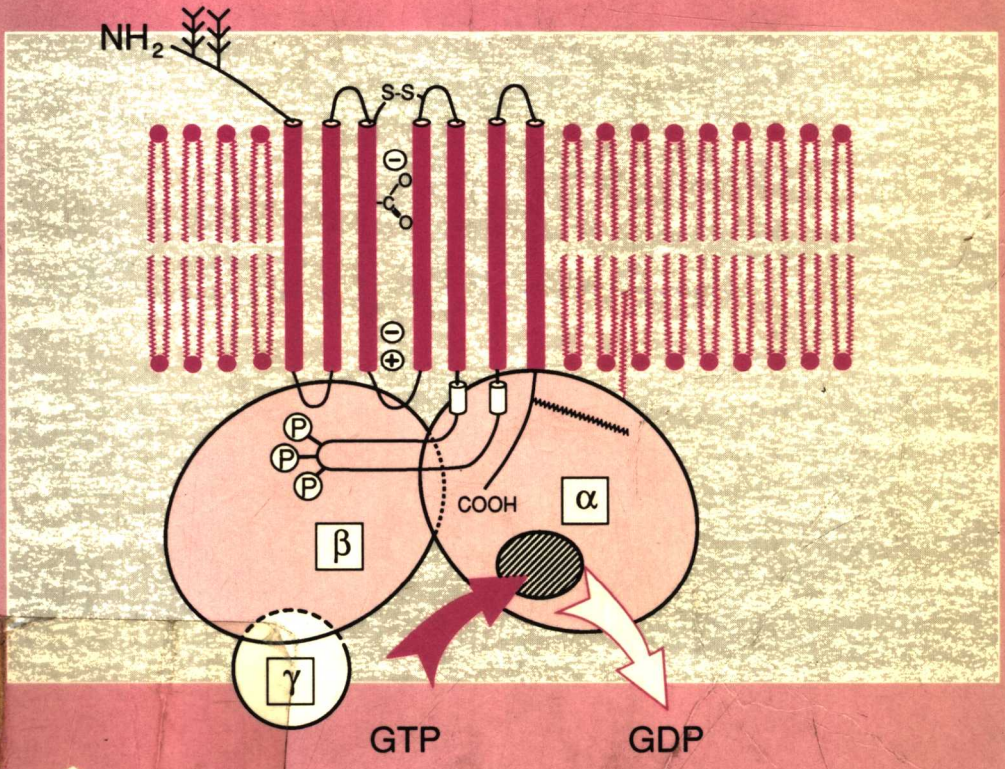
Receptor–Effector Coupling

A Practical Approach

Edited by
E. C. HULME



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Preface

The majority of receptors mediating transmembrane signalling can be classified into three structural and functional categories or groups.

1. Receptors with a single transmembrane segment joining an extracellular ligand binding domain to a cytoplasmic domain. The latter usually has catalytic activity, most often tyrosine kinase, although there are several important exceptions.
2. Receptors which are oligomeric in structure, with each subunit composed of a polypeptide chain having several transmembrane segments. The oligomer bears ligand-binding sites in the extracellular domain, and encompasses a ligand-gated ion channel of defined ionic specificity.
3. Receptors which are monomeric, each molecule being composed of a single polypeptide chain thought to traverse the membrane seven times. This forms a helical cluster which contains a ligand-binding site, and, on the cytoplasmic surface, several loops which recognize, bind, and activate specific heterotrimeric GTP-binding proteins (G-proteins) in an agonist-dependent manner. The activated GTP-liganded G-proteins then mediate a wide variety of events, both at the cell surface by opening specific ion channels, and within the cell by activating enzymes which produce or break down second messengers such as cyclic AMP, inositol trisphosphate, diacyl glycerol, and arachidonic acid.

Companion volumes in this series address the subject of the pharmacological study of receptors through their ligand interactions (*Receptor–Ligand Interactions: a Practical Approach*) and the biochemistry of receptors, particularly their purification and protein chemistry (*Receptor Biochemistry: a Practical Approach*). In the present volume, some important aspects of the study of receptor–effector coupling form the subject matter.

Several chapters are concerned with the study of the effector activities of G-protein coupled receptors. The functions of these receptors are so diverse that the problem is to know where to draw the line. However, the fundamental aspects of receptor–G-protein interaction are surveyed, including the purification and assay of G-proteins (Chapter 1), the detection and characterization of pre-existing receptor–G-protein complexes (Chapter 2), the reconstitution of both cyclase-stimulatory and cyclase-inhibitory receptors with G-proteins (Chapters 3 and 4), including the reconstitution of adenylyl cyclase activity itself, and information on the co-reconstitution of tyrosine kinases, which provides a possible approach to the study of cross-regulation between the signal transduction pathways represented by group 1 and group 3 receptors.

One of the most important signalling mechanisms operated by activated G-proteins is the breakdown of polyphosphoinositides, and the rise in intracellular calcium concentration which is at least partially a consequence of the liberation of inositol trisphosphate into the cytoplasm. Assay methods for inositol phosphates are evolving rapidly. Some of the more recently developed methods are covered in Chapter 5, which also discusses considerations important to radiolabelling of cellular phosphoinositide pools. The measurement of intracellular Ca levels by spectroscopic and electrochemical methods is discussed in Chapter 6. This chapter also contains an introduction to the technology of the control of intracellular Ca levels by the use of photoactivatable 'caged' calcium chelators. This important new technology is also applicable to the inositol phosphates themselves, and to guanine nucleotides. Unfortunately, we were unable to cover this aspect in the present volume.

Both group 2 and group 3 receptors are capable of opening (or in some cases, closing) ion channels. The definition and study of ion channels has been revolutionized in not much more than ten years by the development of the technology of the patch clamp. This has, for the first time, enabled the electrical characteristics and molecular pharmacology of individual channels to be studied. These techniques are now integral to the detection and definition of receptor-operated channels, and the study of their properties and structure–activity relationships, both in *ex vivo* preparations, and using recombinant receptors expressed in cell lines *in vitro*. This important methodology is surveyed in Chapter 7, in as much detail as is possible within the confines of a single chapter.

The effector mechanisms of the group 1 tyrosine kinases are amongst the most important because of their role in controlling cell growth and proliferation but are also amongst the most enigmatic, because the end-effectors are largely unknown. Whilst the enzymatic activity itself is easily measured, the detection of the important substrates whose activity is modulated by tyrosine phosphorylation is a difficult and largely unsolved problem. The detection of tyrosine phosphorylating activity, and the isolation of phosphorylated proteins, is discussed in Chapter 8. This presentation includes important isolation techniques based on the use of anti-phosphotyrosine antibodies which can be used to identify potential substrates.

In addition to the main chapters, a short appendix is provided, which gives an outline of ligand-binding techniques.

Inevitably, in a book of this kind, one is conscious of gaps and omissions. Some of these are due to circumstances beyond the editor's control. Hopefully, they can be remedied in later publications.

Finally, I would like to thank the authors for their fine contributions to this book, and for their understanding, and perseverance.

E. C. Hulme

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Abbreviations

ACh	acetylcholine
ADP	adenosine diphosphate
ALP	alprenolol
α_2AR	α-adrenergic receptor
AM	acetoxymethyl esters
AMF	$Al^{3+}/Mg^{2+}/F^-$
anti-P-tyr	antiphosphotyrosine
AppNHp	adenylylimidodiphosphate
ARF	ADP-ribosylating factor
ATP	adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane *N*, *N*, *N'*, *N'*-tetraacetic acid
βAR	β-adrenergic receptor
BSA	bovine serum albumin
Ci	Curies
CDTA	trans-1,2-diaminocyclohexane-*N*, *N*, *N'*, *N'*-tetraacetic acid
cGMP	cyclic guanosine monophosphate
CHAPS	3-(3-cholamidopropyl)dimethylammonio-7-propane sulfonate
CHAPSO	3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate
CMC	critical micellar concentration
CYP	iodocyanopindolol
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid
FPLC	fast protein liquid chromatography
GDP	guanosine diphosphate
GppNHp	guanylylimidodiphosphate
G-proteins	GTP-dependent regulatory proteins
GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
HEN	hepes/EDTA/NaCl buffer
HPLC	high performance liquid chromatography
IAP	pertussis toxin
Ins 1, 2, 4, 5-P_3	*myo*-inositol 1: 2-cyclic 4, 5-trisphosphate
Ins 1, 3, 4, 5-P_4	*myo*-inositol 1, 3, 4, 5-tetrakisphosphate
Ins 1, 4, 5-P_3	*myo*-inositol 1, 4, 5-trisphosphate
$InsP_1$	inositol monophosphate
$InsP_2$	inositol bisphosphate
$InsP_3$	inositol trisphosphate
$InsP_4$	inositol tetrakisphosphate

$InsP_6$	phytic acid
K	affinity (association) constant
K_d	dissociation constant
KLH	keyhole limpet haemocyanin
KPB	potassium phosphate buffer
LDH	lactate dehydrogenase
LPO	lactoperoxidase
mAChR	muscarinic acetylcholine receptors
MDH	malate dehydrogenase
nAChR	nicotinic acetylcholine receptor
NAG	*N*-acetyl-glucosamine
NEM	*N*-ethylmaleimide
NMR	nuclear magnetic resonance
NMS	*N*-methylscopolamine
Oxo-M	oxotremorine-M
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PEG	polyethylene glycol
P_i	inorganic phosphate
PKC	protein kinase C
PMSF	phenylmethylsulphonylfluoride
PrBCM	propylbenzilylcholine mustard
PtdIns	phosphatidylinositol
PtdIns 4, 5-P_2	phosphatidylinositol 4, 5-biphosphate
PTX	pertussis toxin
QNB	quinuclidinyl benzilate
RTK	receptor tyrosine kinase
$S_{20,w}$	sedimentation coefficient at 20°C in water
SEM	standard error of the mean
SIT	silicon-intensified target
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TED	tris/EDTA/dithiothreitol buffer
TLCK	Na-*p*-tosyl-L-lysine chloromethyl ketone
TLE	thin layer electrophoresis
TPA	O-tetradecanoylphorbol 1,3-acetate
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone
TPEN	*N*, *N*, *N'*, *N'*-tetrakis(2-pyridylmethyl)-ethylenediamine
VIP	vasoactive intestinal polypeptide

Contents

1

Preparation of G-proteins and their subunits

PAUL C. STERNWEIS and IOK-HOU PANG

1. Introduction

The actions of many hormones that interact with receptors on the cell surface are mediated by a family of GTP-dependent regulatory proteins (G-proteins). The G-proteins are stimulated by a host of receptors and regulate a variety of intracellular activities (adenylyl cyclase, phospholipases, ion channels, and a cGMP-dependent phosphodiesterase). Membership in this family is still growing through the use of molecular genetics and more sophisticated techniques for detection and purification of the proteins from diverse sources. The details of the structural complexity and mechanisms of action of the identified G-proteins have been reviewed (1, 2). A brief discussion follows.

All of the G-proteins clearly identified so far contain three subunits (α, β, and γ). The α-subunits are the largest in apparent size ($M_r = 39\,000$–$52\,000$), contain the binding site for guanine nucleotides, are the most diverse, and are thought to define the different G-proteins. The α-subunits also contain the sites for modification by bacterial toxins; however, the existence of hormone-regulated activities that apparently utilize G-proteins but are resistant to intervention with toxins, and the identification by cDNA cloning of an α-subunit (α_z) that lacks a site for ADP-ribosylation (2), suggest that this property is not universal. The β-subunits are less diverse ($M_r = 36\,000$ and $35\,000$ Da) and are believed to be common to the different G-proteins. While there are multiple γ-subunits, little is known about them. The β- and γ-subunits have only been purified in an active form as a non-convalent complex.

The inactive form of the G-proteins contains bound GDP. Exchange of this GDP for GTP results in activation of the G-protein. The proteins possess an endogenous GTPase activity that hydrolyses the GTP and returns the proteins to the inactive state. Thus, the lifetime of an activated G-protein is, at best, limited to its own slow hydrolytic rate. Activation of the G-proteins causes dissociation of the α- from the $\beta\gamma$-subunits. *In vitro*, this activation is accomplished with non-hydrolysable guanine nucleotides or a combination of Al^{3+} and F^-. Under these conditions, the α- and $\beta\gamma$-subunits can be separated. Isolated, activated α-subunits of G_s and G_t (transducin) were shown to be sufficient to activate

adenylyl cyclase and cGMP-dependent phosphodiesterase, respectively. Thus, the α-subunits have been referred to as the activating subunits. Since the $\beta\gamma$-subunits increase the deactivation rate of the α-subunits, they have been proposed to provide an indirect pathway for inhibitory action by the G-proteins. This was specifically defined for the G_i protein(s) which inhibits adenylyl cyclase. In this scenario, $\beta\gamma$ provides a means of communication or concurrent regulation among the G-proteins. The diversity within the structure of $\beta\gamma$ and reports of other potential activities, however, suggest a broader role for these subunits.

The role of the receptor in the activation of G-proteins is the stimulation of guanine nucleotide exchange. Stimulation of GDP release results in binding of GTP, activation of the G-protein, and subsequent regulation of effector enzymes. The receptors accomplish this in a catalytic fashion and thus amplify the signal of the hormone. Where examined so far, the interaction of receptors with G-proteins requires the holo-protein ($\alpha\beta\gamma$). The use of G-proteins in studies of reconstitution with receptors is covered in other sections of this book (see Chapters 3 and 4).

The extent of hormonal regulation that utilizes G-proteins is still being determined. Current questions are many. For example, how many unique G-proteins exist? Which receptors work through the G-proteins and what is their specificity of interaction? What pathways for intracellular signalling are regulated by G-proteins? Which G-proteins effect regulation of the different pathways? What are the detailed mechanisms of action for signalling via G-proteins? Purification of the G-proteins and their reconstitution with other components is one approach for the exploration of these and other questions.

This chapter presents methods for the detection and isolation of G-proteins, with primary emphasis on preparation of the proteins from brain. These methods have proven successful for other tissues in a variety of animals; they have even been applied successfully to eggs from the sea urchin. The procedures have been developed in a number of laboratories. We have used all of the techniques. The descriptions herein present the details of their application in our hands.

These methods result in the isolation of at least five unique G-proteins from the bovine central nervous system. In summary, these are G_s, two G_i proteins, G_o, and G_t (transducin). G_s is the G-protein that stimulates adenylyl cyclase. A G_i protein was first described as a mediator of inhibition of adenylyl cyclase. However, at least three $G_i\alpha$-subunits have been identified by homology of their deduced sequences. G_o is the most abundant G-protein in brain. The G_i and G_o proteins have been implicated in the regulation of phospholipases and ion channels but their specificity of action remains to be determined. Finally, G_t is a specialized G-protein, found in retina, that stimulates a cGMP-dependent phosphodiesterase in response to activation of rhodopsin by light. G_t is obtained from retina in a simple procedure which is described briefly. The other G-proteins require extensive purification which is described in detail.

Three procedures are commonly utilized to detect the G-proteins, binding of [^{35}S]GTPγS, ADP-ribosylation by bacterial toxins, and stimulation of adenylyl

cyclase (G_s only). These are discussed in Section 3. The future will, no doubt, provide more specific functional assays and simplified detection with specific antibodies (now being developed). The expression of the G-proteins in bacteria or yeast through molecular genetic techniques and the purification of these proteins is developing rapidly and will provide abundant and specific sources for those proteins or subunits that occur in lesser quantities (21, 22).

2. Procedures for purification of G-proteins

2.1 Materials

This section discusses two reagents tht require preparation prior to initiation of the procedures listed below. A list of common stock solutions is also presented. Distilled or highly deionized water is used for all solutions.

2.1.1 Purification of cholic acid

This anionic detergent is purchased from Sigma but can be obtained from other vendors. It produces a very deep yellow to brown solution. The colour is from impurities which are mostly anionic and render DEAE gels black during use. It is purified as follows.

Protocol 1. Purification of cholic acid

1. Prepare a 400 ml column of DEAE Sepharose or DEAE Cellulose.
2. Dissolve 500 g of cholic acid (1.25 mol) in 3 litres of water. Since the acid is not soluble, initially add 100 ml 10 M NaOH and stir for several minutes. Then add more of the base to adjust the pH to 7.5–8.0 and stir while the cholic acid slowly dissolves and the pH drops. Repeat until all of the cholate is dissolved and the pH has stabilized.
3. Dilute to 20 litres with water to give a 2.5% w/v solution. Pass the solution through the DEAE matrix. Most of the impurities bind tightly and turn the top of the matrix black. The cholate flows through the column after equilibrating the binding sites. Wash the column with 2 litres of water.
4. Acidify the filtrate in large beakers or buckets (5–10 litres). While mixing, slowly add 10 N HCl. The cholic acid precipitates as white chunks. Add acid with vigorous stirring until the pH drops to 4 and remains constant.
5. Collect the precipitate by filtration with vacuum. Wash with 1 litre of water to remove salts. In a hood, wash with 200 ml anhydrous ether to help extract any undesired lipids such as cholesterol and help dry the preparation.
6. Air-dry for 1 day in a fume hood to remove ether. Powder the precipitate and dry under vacuum with or without low heat (40 °C) until the weight is constant. Store at room temperature in tightly capped containers. The yield is about 90%.