1

The components and foundations of signalling

Endocrine glands produce hormones that act as *first messengers*, informing other tissues about the external environment, overall energy status, etc., on a strictly 'need-to-know' basis. Tissues that do not need to respond to a given first messenger may lack a functional receptor or pathway for that signal. First messengers (except steroids) cannot enter the cell, and so elaborate ways of transducing the signal to the intracellular compartments must be employed. Some receptors couple with an effector enzyme that produces a *second messenger* inside the cell. These second messengers are almost invariably charged or membrane bound and therefore cannot leave the cell. The final stages in the transduction of the signal usually involve the activation of downstream protein kinase enzymes, often by direct binding of the second messenger. Downstream kinases are mostly serine/threonine kinases and they activate (or inactivate) target proteins by phosphorylation.

The biological response that a cell makes to a received signal can also vary. cAMP-inducing catabolic signals such as adrenaline activate glycolysis in the heart but have an opposite effect in the liver. This is due to the differential effects of PKA phosphorylation on the different isoforms of an effector enzyme that either makes or breaks down the second messenger, fructose-2,6-bisphosphate. In most tissues, insulin is strictly anabolic, inducing target cells to incorporate glucose into macromolecular stores. In the heart, however, insulin uniquely stimulates glycolysis.

Structure and Function in Cell Signalling by John Nelson

A multicellular animal (*metazoan*) faces a major problem not shared by unicellular forms (*protozoans and prokaryotes*) namely, how to integrate, organise and control the dynamics of the diverse collection of differentiated tissues and organs that make up a body. The most obvious means of control, of course, is the central nervous system: one wills a skeletal muscle to move, and it moves. However, much of the signalling between tissues is done entirely unconsciously and it is this unconscious signalling that controls vital processes such as growth, reproduction, food and energy usage, host defence and immunity, fight or flight responses and many other day-to-day functions that one tends to take for granted. Some of these latter signals are initiated by the autonomic nervous system; others are entirely hormonal with no conscious or unconscious neuronal input.

In its widest definition, *cell signalling* encompasses the generation and transmission of a signal (in the form of a blood borne first messenger, for example), the reception of the signal (by the target cell's receptor) and the propagation of that signal (via second messengers, for example) within the receiving cell. *Signal transduction* is the process whereby an extracellular signal is converted (or *transduced*) into a different form (or forms) of intracellular signal. Transduction of the signal often results in amplification and is frequently a multi-step process. Crucially, these processes are time-limited. In other words, a signal is only allowed to be transduced for a certain length of time before it is turned off.

1.1 Definition of terms used

1.1.1 First messengers

A first messenger is an extracellular molecule that is recognised by target cells because they possess receptors that bind the molecule specifically. This is how cells can communicate with each other – often over long distances. Cells that should not respond to a particular first messenger will lack the matching receptor. However, signal transduction in responsive cells can be still be attenuated by either down-regulating the number of receptors per cell, up-regulating an enzyme that inactivates the first messenger, or desensitising the downstream intracellular transduction pathway. There are hundreds of distinct first messengers ranging in size from large glycosylated polypeptide hormones such as gonadotrophin, through polypeptides, tripeptides, cholesterol-like steroids to amino acid derivatives (Figure 1.1). Smaller messengers are the neurotransmitter amine acetyl choline and even smaller paracrine 'hormones': the gaseous hormones, nitric oxide and hydrogen sulphide.

1.1.2 Glands and types of secretion

Glands can be divided roughly into *exocrine* and *endocrine*. Exocrine glands secrete into the gastrointestinal tract or onto the surface of the body (Figure 1.2). Exocrine

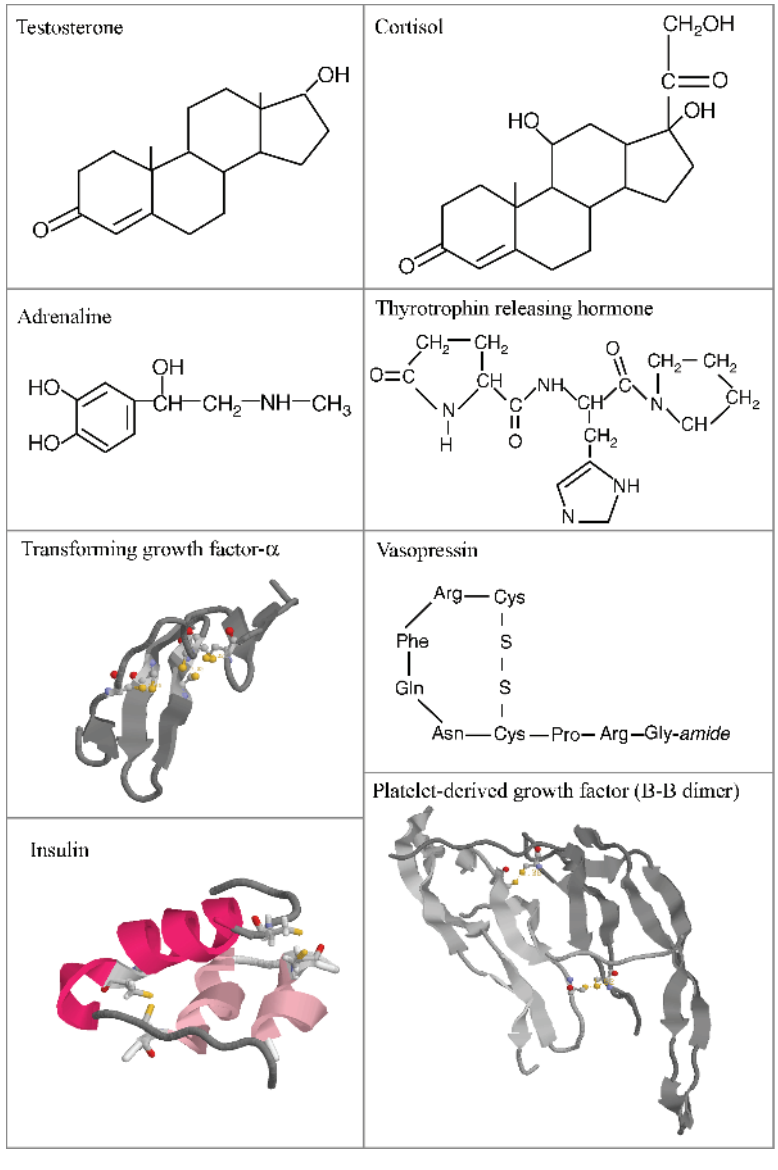

Figure 1.1 First messengers

secretions generally play a digestive or protective role and are not involved in signalling. Endocrine glands, on the other hand, secrete informational molecules into the blood system. A few organs, such as the pancreas, contain both exocrine and endocrine compartments. Not all first messengers travel through the blood; some act locally or even self-stimulate via autocrine secretion (see Section 10.3) (Figure 1.3).

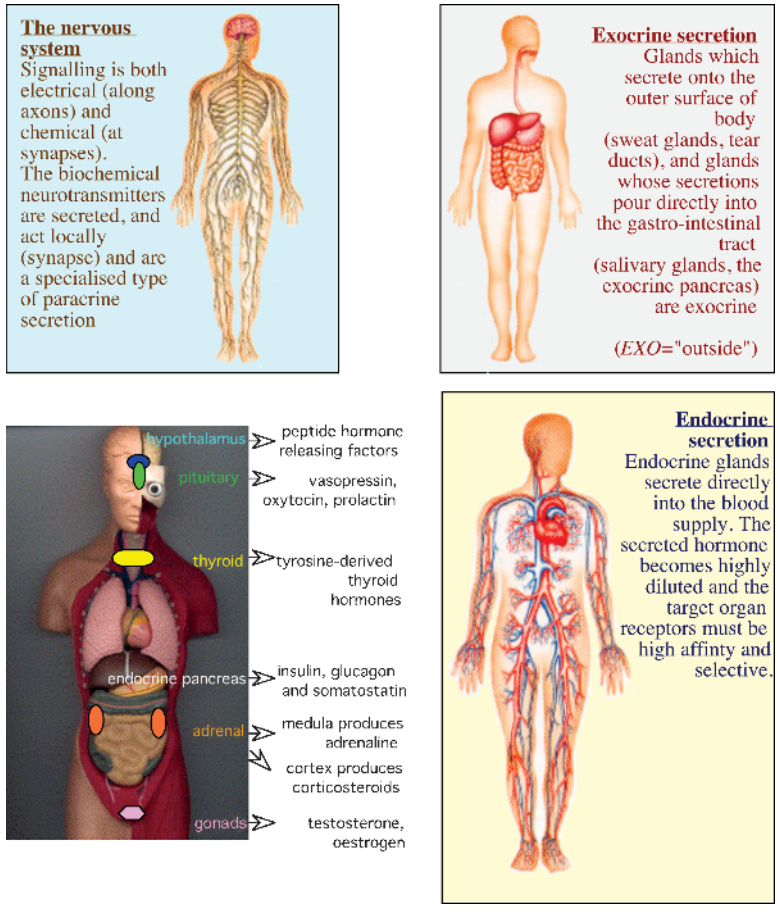

Figure 1.2 Glands

1.1.3 Ligands

All first messengers are 'ligands', which simply means: a molecule that binds to a given receptor protein in a specific and saturable manner. Second messengers are also ligands, but for intracellular receptors.

1.1.4 Agonists

Agonists are ligands that cause a biological response when they bind to their cognate receptor. They include:

- the natural hormone (or first messenger);

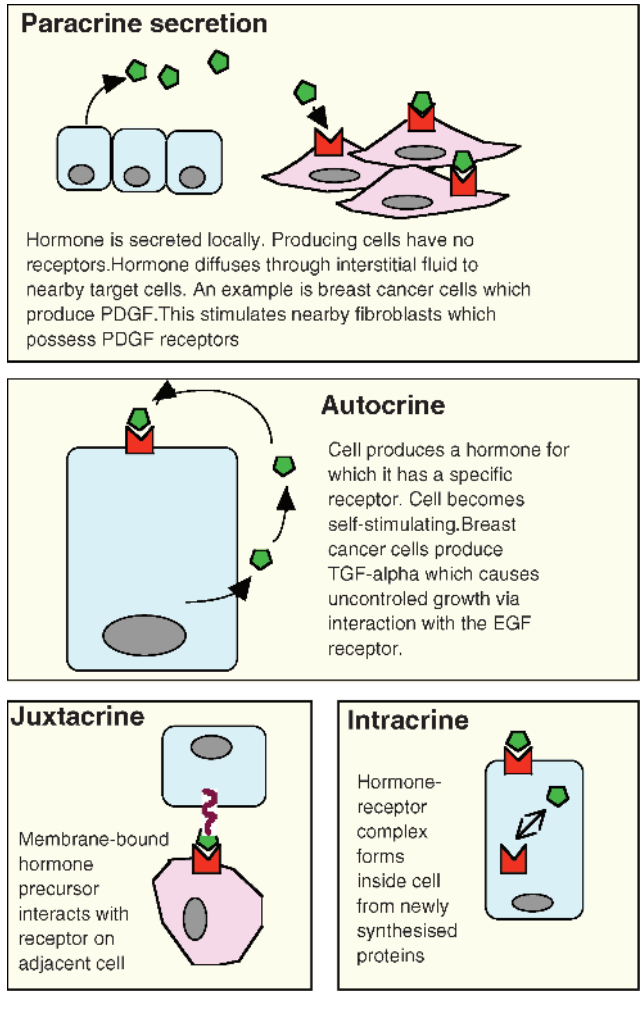

Figure 1.3 Informational molecules do not all travel through the circulation

- synthetic analogues (or drugs) which produce a similar response when interacting with the receptor in question – for example, adrenaline is the natural agonist, and the analogue isoproterenol an artificial agonist, of the β-adrenergic receptor.

1.1.5 Antagonists

Antagonists are also ligands, which bind specifically and saturably to a given receptor but without producing a cellular response. Thus, antagonists occupy the receptor, effectively blocking the activating effects of the natural first messenger. Almost all antagonists are human-made. Very few natural ligands are true receptor antagonists.

Table 1.1 Ligand types

Conventional agonists	Full agonist	At full receptor occupancy, elicits full biological response
	Partial agonist	Even at saturation, only a partial biological response is elicited‡
	Inverse agonist	Binds to, and stabilises the inactive form of the receptor (or converts R* to R)§
Antagonists	Competitive antagonist	Binds to the same site on the receptor as the agonist and blocks its effects
	Noncompetitive antagonist	Binds to a different site on the receptor than the agonist and reduces its effects

‡ Critically depends on system tested
§ See Chapter 2

Blocking receptor pathways in nature almost always comes about by indirect antagonism from another pathway.

It is likely that most polypeptide antagonists bind unproductively to the same site on the receptor that would be occupied by the native agonist. However, synthetic catecholamine ligands fall into many subcategories (Table 1.1). Undoubtedly, this multiplicity was discovered because of the huge research effort inspired by the clinical importance of these receptors as drug targets, the ease of synthesis of such small ligands, and the availability of simple *in vitro* screens. Whether receptors for large complex ligands such as glycoproteins also display such diverse behaviour is open to question. However it is possible given that insulin & IGF-1 bind to different domains of InsR Ectodomain (see Chapter 2, Section 2.3.3).

1.1.6 Receptors for first messengers

The receptors we are concerned with are large proteins that 'receive' information in the form of first messengers. 'Reception' is by direct binding of the hormone to a specialised ligand-binding site on the receptor. Other 'receptors' can act both as information receivers and attachment factors such as the integrins that bind cells to basement membranes and participate in platelet activation. In the broadest sense, the term 'receptor' also includes *non-informational* receptors such as endocytic or scavenger receptors. These receptors (the liver's asialoglycoprotein receptor is an example) serve a waste-disposal role in the body, mopping-up aged proteins from the circulation – they do not 'receive' information.

First messenger receptors fall into two broad categories: soluble or membrane-spanning. Intracellular soluble receptors are members of the 'zinc-coordinating domain' superfamily of transcription factors. Being transcription factors, they are largely nuclear in location. They include receptors for lipophilic hormones and include oestrogen-, androgen-, and progesterone-receptors, as well as thyroid hormone receptors and those for retinoids and vitamin D3 analogues.

Membrane-spanning receptors are multi-subunit, single-pass, or 7-pass (Figure 1.4). The multi-subunit receptors are ligand-gated ion channels that allow specific ions to cross the membrane when agonist-occupied, through a central pore that closes when the

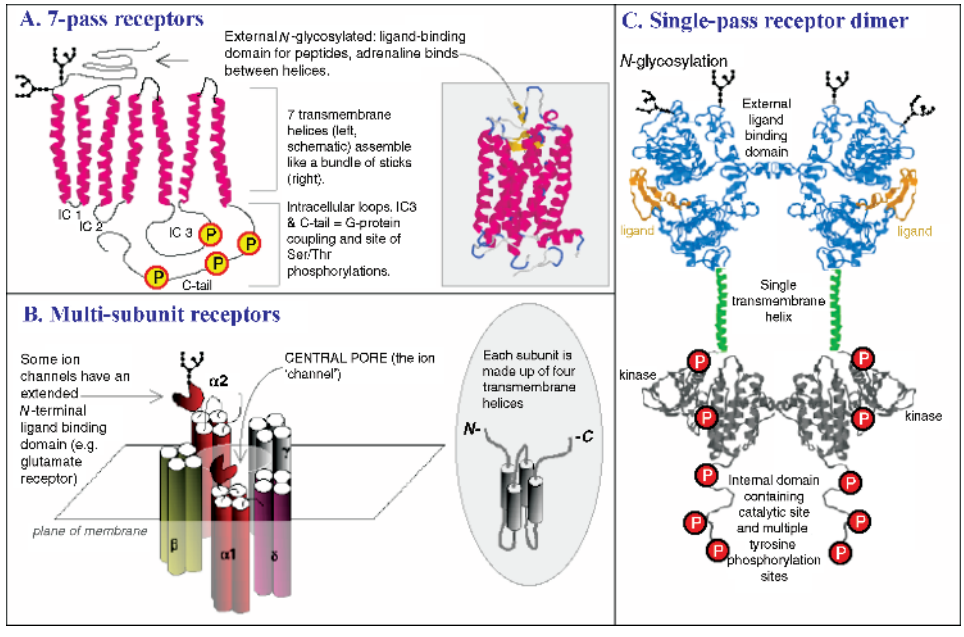

Figure 1.4 Schematic cartoons of some membrane-spanning receptor types

receptor is unoccupied. These are a numerous and diverse group of fast-acting switches that are typically made up of four or more subunits that are usually (but not always) products of more than one gene. The nicotinic acetyl choline receptor, for example, is made up of five subunits: two ligand-binding α-subunits and one each of β-, γ- and δ-subunits. The central pore is Na^+-selective and, when activated, extracellular sodium ions are allowed into the cell, causing local depolarisation of the membrane and activation of voltage-gated channels.

Unfortunately, space does not permit a detailed discussion of these receptor types and only the IP3 multi-subunit receptor will be mentioned in the text.

Receptors that have a single transmembrane helix, I shall term 'single pass'. These are either catalytic or non-catalytic. Non-catalytic single-pass receptors rely on recruitment of cytoplasmic proteins (often tyrosine kinases) to transduce their signals. These include the 'tumour necrosis factor' receptor and the 'cytokine receptors'such as those for the interleukins. Again, space does not permit a detailed discussion of non-catalytic single-pass receptors.

Catalytic single-pass receptors are predominantly tyrosine kinases and can be phylogenically connected through catalytic domain homologies (see Appendix 1). A smaller group are serine/threonine kinases, including the growth-inhibitory 'transforming growth factor-β' receptor.

The receptor tyrosine kinase class of single-pass receptors play major roles in all aspects of metazoan life and, being the best understood, are one focus of the present text. Receptor tyrosine kinases, activated by polypeptide 'growth factor'-type ligands, phosphorylate specific intracellular substrates, and are characterised by themselves

becoming tyrosine-phosphorylated. They dimerise when ligand-activated and phosphorylate each other in the first steps in a signal transduction programme.

The evolutionary more ancient 7-pass receptors are non-catalytic, phylogenetically linked and the single largest receptor superclass. They often become serine/threonine-phosphorylated after signalling has peaked.

1.1.7 Second messengers

Second messengers are confined within the cell that synthesised them because all are either charged (and thus impermeant to the cell membrane) or are incorporated into the inner leaflet of the plasma membrane. For example, although sometimes secreted by social amoeba, cAMP release from a cell is only possible when the membrane is ruptured, and extracellular cAMP is thus a sign of cellular damage.

1.1.7.1 A few types of second messenger

Second messengers are generally smaller than first messengers and there are far fewer individual types (Figure 1.5). Discrimination in cellular information processing is concentrated upon the large diversity of hormone receptors and extracellular cognate ligands. Once the signal is transduced into the cell, the options are reduced. Many different receptors may be capable of stimulating production of a common second messenger.

Second messengers are produced by 'effector enzymes' downstream of extracellular receptors. The effector enzymes responsible for the synthesis of second messengers are

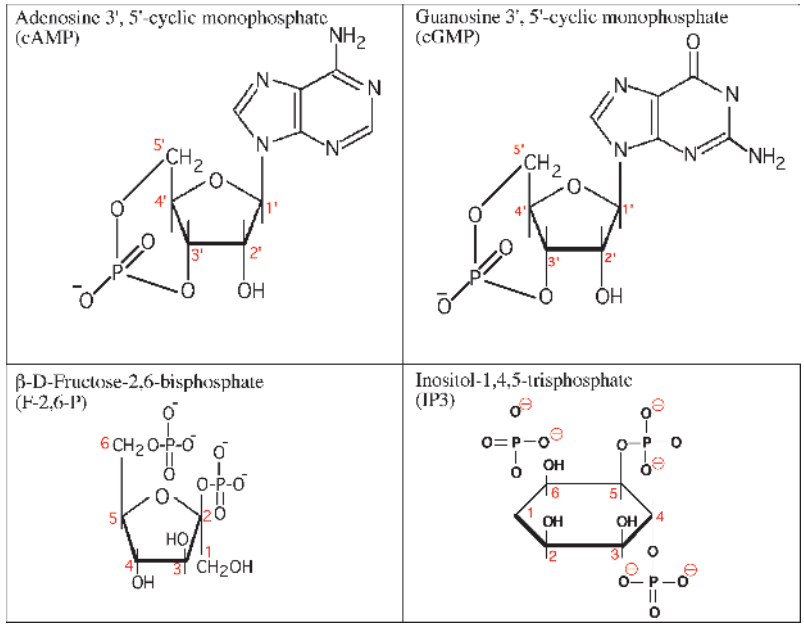

Figure 1.5 Second messengers

under tight control, only being active for short bursts. Crucially, transduction systems have the means to destroy the second messengers or sequester them when the signal needs to be terminated.

An archetypal system is represented by the effector enzyme adenylyl (or adenylate) cyclase that produces cAMP, which is in turn broken down by the signal-terminating enzyme phosphodiesterase.

1.1.8 Soluble second messengers

cAMP The cyclic nucleotides cAMP and cGMP (Figure 1.5) are produced by cyclase enzymes that use ATP or GTP as substrates. 'Adenyate' or adenylyl cyclase enzymes are a diverse family of membrane-spanning proteins. In mammals they have a 'split' catalytic that is re-united upon stimulation by a variety of upstream signals.

Calcium Calcium release from intracellular stores in the endoplasmic reticulum lumen is controlled by ligand-gated ion channels that are receptors for inositol-1,4, 5-trisphosphate (IP3). Free calcium is maintained at a low level (around 150 nM) in the cytosol of resting cells against a high extracellular concentration of 1–3 mM in plasma (Figure 1.6). This low level is maintained by highly active calcium pumps that either expel the ion to the extracellular space, or channel it into internal storage in the endoplasmic reticulum. However, when IP3 binds to its receptors in the endoplasmic reticulum membrane cytosolic calcium rises rapidly to around 1 μM. IP3 receptors are calcium-selective channels that open to release the ion into the cytosol. Other sources of calcium come from cell surface 'store-operated' channels that permit extracellular calcium to refill the internal stores as well as contributing to certain types of signalling. Finally, membrane depolarisation downstream of plasma membrane ligand-gated sodium channels (like the nicotinic acetyl choline receptor) also causes external calcium entry via voltage-gated calcium channels.

Much of calcium's effects are mediated by binding to the ubiquitous calcium-binding protein, calmodulin, various forms of calcium-dependent protein kinase (PKC) and the multi-subunit phosphorylase kinase.

Fructose-2,6-bisphosphate Fructose-2,6-bisphosphate (F-2,6-P) is produced by phosphofructokinase-2 (PFK-2). It should not be confused with fructose-1,6-bisphosphate (F-1,6-P). F-1,6-P is a key intermediate in the energy-harvesting glycolysic pathway, whereas F-2,6-P plays no part in energy-generation but instead acts solely as a second messenger.

1.1.9 Membrane-bound second messengers

Like soluble second messengers, the key to signalling lipids is that they normally are absent, or at a very low level, in the resting cell membrane. Phosphatidyl inositol-4,5-bisphosphate (PIP2) is an important lipid substrate for a variety of effector enzymes.

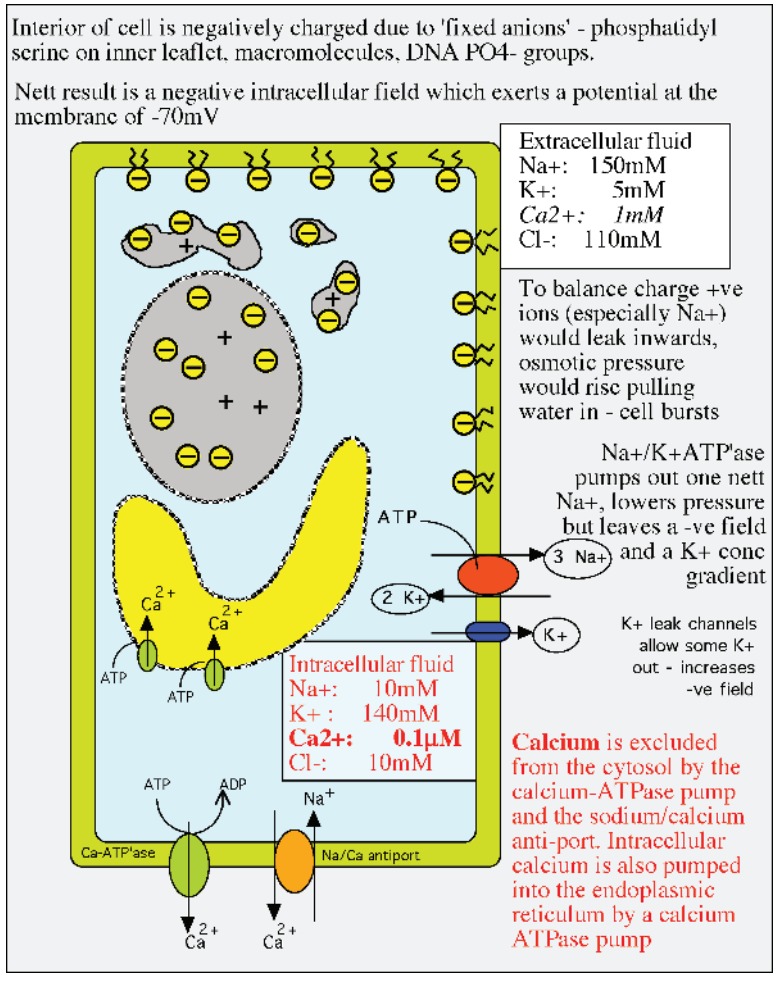

Figure 1.6 Summary of ionic homeostasis

Phospholipase C signals – diacylglycerol and IP3 Diacylglycerol (DAG) is the product of PIP2 hydrolysis by signal-activated phospholipase C (PLC). PIP2 distribution is asymmetric: it is only found on the inner leaflet of the plasma membrane. PLC cleavage of PIP2 yields two second messengers – the soluble hexitol IP3 and the membrane-bound DAG – which are produced simultaneously (Figure 1.7). There is a family of these signal-activated phospholipases, including phospholipase A and phospholipase D, each with a characteristic cleavage each site in PIP2.

PI-3-kinase signals – PIP3 Phosphatidyl inositol-3,4,5-trisphosphate (PIP3) is the product of the enzymic activity of an important family of signal-activate lipid kinases that also use PIP2 as a substrate. Like DAG, PIP3 is practically absent in the plasma membrane of resting cells.

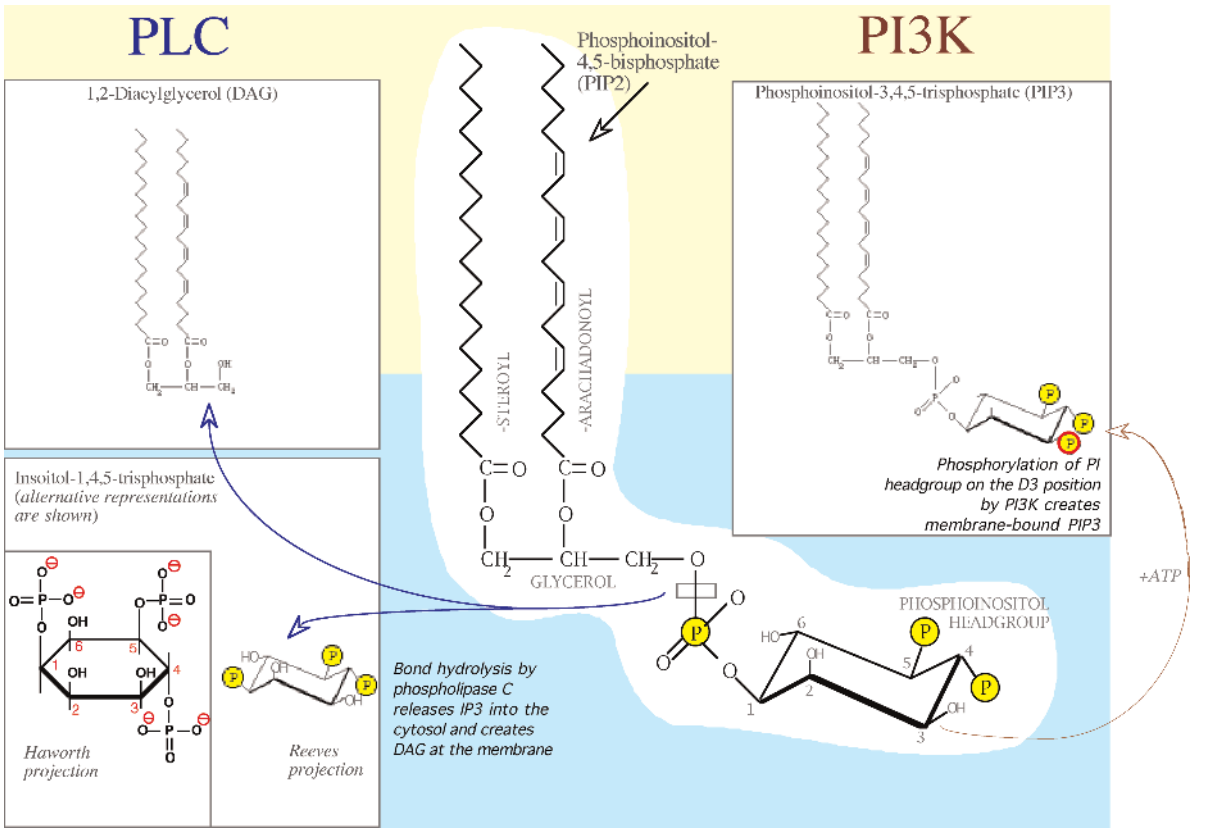

Figure 1.7 Two major signalling pathways utilise the same membrane bound substrate, PIP2

Phosphoinositide-3-kinase (PI-3-kinase) family members are downstream effectors of both single-pass and 7-pass receptors and all produce the activating 3-phosphorylation of the PI headgroup.

The PI-3-kinase family is divided into three classes[1]. Class 1 PI-3-kinases use PIP2 as a substrate and produce PIP3 in response to signals from single-pass receptor tyrosine kinases or heterotrimeric G protein β/γ subunits.

Class 1A are heterodimers consisting of a large, 110 kDa, catalytic subunit (p100α, p110β or p110δ) and a smaller regulatory subunit that processes the upstream signal. The catalytic subunits possess Ras-binding activity. Class 1A regulatory subunits, of which several splice variants exist, are of two basic types: p85 and p55. Both types contain a tandem pair of SH2 domains on either side of an interaction domain that binds the catalytic subunit. The smaller p55 subunits have lost an *N*-terminal regulatory region present in p85 that consists of an SH3 domain, polyproline segments and a domain resembling a Rho/Rac-GAP (see Chapter 3).

Class 1B PI-3-kinases are typified by the p110γ catalytic subunit. This class of PI-3-kinases do not interact with SH-2 containing adaptors but instead have a unique binding partner (p101). Class 1B types are activated by free β/γ subunits released from activated G proteins and it is the p101 regulatory subunit that mediates this activation[2].

Like the Class 1A p110α catalytic subunits, p110γ also has a Ras-binding domain and it has been shown that PI-3-kinase may act as an effector of Ras. Binding of activated Ras<GTP> increases PI-3kinase activity by 8 to 20-fold[2].

Class II PI-3-kinases do not use PIP2 as a substrate, but instead use phosphatidyl inositol-4-phosphate. They are >200 kDa and contain a calcium-binding C2 domain. Class III types use phosphatidyl insositol only.

1.2 Historical foundations

> Fortune favours the prepared mind
>
> Louis Pasteur

I include the following brief notes on early experimental milestones for three reasons. First, early serendipitous findings led to protocols still in use today and it is worth knowing where some of these odd reagents and methods came from. Second, these founding episodes illustrate the passion, persistence, good and bad luck, controversy and sheer bloody-mindedness that was (and still is) part-and-parcel of the field. Third, the way that hypotheses were set up, and then knocked down on the road to clarity, is a good exemplar of the scientific method. Even after a century, some important areas of signalling are still mysterious and many paradoxes remain unresolved.

1.2.1 When did the discipline of cell signalling begin?

One could argue that the discipline was born out of the furious scientific debates ignited by the work of the Russian physiologist, Ivan Petrovich Pavlov, and his discovery of the 'conditioned reflex'. Pavlov was the first to reveal the workings of the autonomic nervous system in his famous demonstration that dogs, conditioned to expect food at

the sound of a bell, would salivate simply at the sound, even without food being presented. He predicted that the autonomic nervous system would be found to be responsible for all aspects of control of digestion: In other words, (unconscious) neuro-electrical control solely governed gastrointestinal secretions, in particular initiation of exocrine pancreatic secretion after a meal, which he had shown was dependent upon vagal nerve stimulation.

1.2.2 The discovery of 'hormones' – Bayliss and Starling, 1902

In a lab in University College London, on the afternoon of Wednesday, 16th January 1902, the English physician Ernest Henry Starling and his physiologist colleague William Maddock Bayliss proved Pavlov wrong and in the process discovered *secretin*, the first known signalling molecule. Having dissected all nervous tissue from the exposed jejunum of an anaesthetised dog, leaving only connected blood vessels, they found that merely stimulating the denervated jejunum with dilute hydrochloric acid prompted pancreatic secretion even though there was no neuronal connection between the two tissues. Pavlov's theory held this to be impossible.

At this discovery, Starling declared it to be a 'chemical reflex' and immediately improvised a protocol that proved the signal from the intestine had arrived at the pancreas through the blood, rather than the nervous system. Starling excised a section of the jejunum, treated it with HCl, filtered the resulting mucosal secretion and injected the filtrate into the jugular vein of the anaesthetised dog. A friend, Charles J. Martin, who 'happened to be present' recounts that 'after a few moments the pancreas responded by a much greater secretion than had occurred before'. Our witness further remarked that 'it was a great afternoon'.

In 1902, Bayliss and Starling named the jejunal factor 'secretin' and reasoned correctly that it exists, stored in the jejunal mucosa, as an inactive prohormone: *prosecretin*. In 1905, Starling introduced the term 'hormone' (from the Greek *hormao*, meaning 'arousing, initiating or exciting agent') to describe blood-borne substances (chemical first messengers) that initiate such 'chemical reflexes'.

This novel hypothesis could not be accepted without experimental replication. But, in Russia, Starling's chemical stimulation experiment did not work and in London, Pavlov's vagal nerve stimulation could not be reproduced. After some contention, honour was satisfied – experimental protocols were at fault. Neuronal stimulation could not be demonstrated in London because the dog was treated with morphine prior to anaesthesia, whereas Pavlov's group discovered that they had destroyed the secretin by 'over-neutralising' the HCl extract. Both were correct. Neuronal and hormonal control of gastrointestinal secretions are alternative, complimentary systems. Thus began the discipline of 'endocrinology'[3].

Bayliss and Starling are generally credited with the discovery of the first hormone, but the 'suprarenal gland' hormone, *adrenaline*, was actually discovered earlier (in 1901) and its total synthesis achieved in 1904[3]. Starling appeared unaware of this at the time of his lectures of 1905. The 'suprarenal' gland (meaning *above the kidney*) is now known as the adrenal gland.

In the following decades, many more hormones were discovered but most were peptides whose structures would not be elucidated until the 1950s. Identification of their receptors would have to wait until the 1970s.

1.2.3 The discovery of insulin and the beginning of endocrine therapy – Banting and Best, 1921

Insulin was discovered and first purified by Frederick Grant Banting and Charles Best in the labs of Dr. J.J.R. MacLeod at the University of Toronto in 1921, and later shown to rescue patients with diabetes in early 1922. Much later, in 1951, insulin was the first polypeptide to be sequenced (by Fred Sanger and Hans Tuppy) and, later still, the first to be synthesised in the lab.

1.2.4 Peptide sequencing – Fred Sanger, 1951

It is impossible to exaggerate Fred Sanger's importance in modern biochemistry. Although Sanger was not directly involved in signalling or molecular genetics, these fields of research would not exist without the sequencing techniques that Sanger developed; first peptide sequencing in the 1950s and later oligonucleotide sequencing in the 1970s. In recognition of these revolutionary pieces of work, he was awarded two Nobel Prizes.

Sanger did more than provide the world with sequencing techniques. He also dispelled the prevailing notions of what made up proteins. At the beginning of the 1950s, proteins were considered to be mixtures of substances lacking unique structure, whose amino acid compositions were not directly encoded, but rather 'managed' by genes. However, clues that specific amino acids had singular roles to play in proteins were emerging, in particular the finding that a single amino acid substitution in haemoglobin could give rise to sickle cell anaemia. Sanger chose insulin as a target for his first foray into protein sequencing because it was medically important and was available in large quantities and high purity[4]. His technique depended on *N*-terminal labelling with fluorodinitrobenzene (FDNB), which results in covalent addition of dinitrophenyl (DNP) groups to any free amino groups. DNP-labelled peptides are yellow and can be easily identified after separation by chromatography. Proteolytic cleavage combined with partial acid hydrolysis of the peptide (which more-or-less randomly cleaves peptide bonds, yielding cleavage products with new amino termini) and specific proteolytic cleavage, the peptide sequence could be reconstructed from the amino acid compositions of overlapping sequences. Here again, a spin-off was the first proof that the proteases Sanger used (trypsin and chymotrypsin, primarily) were both irreversible and truly specific: *trypsin* cleaving after basic amino acids only (lysine, arginine), and *chymotrypsin* cleaving after aromatic amino acids only (tyrosine and phenylalanine).

Although Sanger's peptide sequencing was largely replaced by automated Edman *N*-terminal degradation – only possible by the later development of high performance liquid chromatography – trypsin and chymotrypsin are still used to fragment proteins for identification by mass spectrometry. Information available from the various genome

projects allows one to identify the trypsin cleavage sites in all known proteins. Each protein will yield predictable and unique trypsin cleavage fragments with unique molecular weights and such patterns can be used to identify the protein (as long as post-translational modifications, such as phosphorylation, are taken into account).

1.2.5 Discrimination of beta- and alpha-adrenergic responses – Ahlquist, 1948

Although adrenaline (also known as 'epinephrine') was long known to have neurotransmitter and hormone-like activities, its diversity of functions was not properly understood until the work of Raymond Ahlquist[5]. Before this seminal work (sadly ignored for five years after its publication), adrenaline was thought to act through either 'excitatory' or 'inhibitory' receptors. By careful analysis of tissue-specific responses to adrenaline, and related agonists and antagonists, Ahlquist confirmed that two distinct receptors existed, but realised that the division into 'excitatory' or 'inhibitory' was too simplistic.

He gave the name 'beta-adrenotrophic' to the receptor mediating adrenaline's 'inhibitory' effects on blood vessels (vasodilation) and uterus (relaxation) as well as its 'excitatory' effect on the heart (myocardial stimulation). The 'alpha-adrenotrophic' receptors, then, were those responsible for 'excitatory' effects on blood vessels (vasocontraction) and uterus (muscular contraction) as well as the 'inhibitory' effect on intestine (relaxation).

Alquist's proof lay in the fact that the alpha responses to adrenaline (both 'excitatory' and 'inhibitory') could be blocked by addition of the antagonist ergotoxine. Yet this alpha-blocking agent had no effect upon beta type responses. Furthermore, the rank order of potency of the known agonists differed between the two types.

Although such receptors remained 'hypothetical structures or systems' until the 1970s, the pharmacological distinction between alpha and beta responses greatly assisted the development of adrenergic-targeted drugs such as 'beta-blockers' (such as propanolol) and laid the groundwork for the later subdivision of adrenergic receptors into the multiple forms known today.

1.2.6 'Acrasin' = cAMP – the ancient hunger signal

In 1947, J.T. Bonner discovered 'acrasin', a name he gave to a diffusable soluble chemical that caused the usually solitary amoeba of the slime mould *Dictyostelium discoideum* to swarm together to form a temporary multi-cellular slug in times of 'famine'. Some 20 years later, it was realised that 'acrasin' was in fact cAMP[6]. Social amoebae such as *Dictyostelium* are the only organisms known to secrete cAMP deliberately, using it as both extracellular first messenger as well as intracellular second messenger[7]. In all other organisms, cAMP is locked within the cell that produces it and acts solely as an intracellular second messenger, only escaping as a result of cellular damage.

1.3 Early milestones in signal transduction research

1.3.1 Cell-free experiments and the discovery of cAMP – Sutherland, Rall and Berthet, 1957

Arguably, the most important early milestone in signal transduction was the discovery by Earl Sutherland of the first 'second messenger': the cyclic nucleotide cAMP[8]. Almost as important, however, was the group's success in working with homogenised cells and purified subcellular fractions, thus disproving a prevailing consensus that cells must be intact for hormonal action to be manifest.

It is important to realise that in the early 1950s the cell was thought of as a mechanism too complex to be disassembled. To quote Sutherland:

> When I first entered the study of hormone action, some 25 years ago, there was a widespread feeling among biologists that hormone action could not be studied meaningfully in the absence of organised cell structure.[9]

Sutherland, and colleagues Jacques Berthet and Theodore Rall, began a series of experiments in November of 1955 aimed at understanding how the hormones adrenaline and glucagon activate the liver enzyme, glycogen phosphorylase[10]. They were favoured by complementing expertise and a measure of good luck. As Theodore Rall comments, 'I have lost count of how many wrong ideas got us to do the right experiments'. Perhaps the most significant happenstance was that Earl Sutherland's laboratory used dogs, rather than rats, as a source of tissues. This was fortunate because his experiments might not have worked otherwise, but unfortunate because most other labs used liver from young male rats.

Their work was greatly simplified by the fact that cAMP is heat-stable. Their final piece of good luck was that the ATP they used was impure – it contained small amounts of GTP. Without this contaminant, their experiments would not have worked, for reasons that would not become clear for another 20 years.

1.3.2 Fluoride – a stimulator of G proteins

By the early 1950s it was known that treating slices of dog liver with adrenaline caused glycogen to be broken down into glucose because the enzyme *glycogen phosphorylase* (GP) was somehow activated. Glucagon or adrenaline could activate GP in liver slices, but not in homogenised liver. Perhaps something liberated by homogenisation was inhibiting the reaction . . . .

Sutherland was able to isolate activated GP from liver slices and show that it could be inactivated by an enzyme that later proved to be a *serine/threonine protein phosphatase*. The phosphatase was found to be inhibited by the *fluoride* ion and so this was routinely added to their incubation mixtures in the expectation that this would allow the GP activation to proceed in homogenates. Sensibly, they always included fluoride-free controls[9]. Had they not done so, the hormonal effects would actually have been masked, because fluoride is capable of *directly* stimulating the G proteins involved in adrenaline and glucagon signalling. This is discussed further in Section 1.4.4.

1.3.3 ATP and subcellular fractionation

Taking a cue from the work of Krebs and Fischer, Sutherland's team were able to restore hormonal sensitivity to homogenates by adding ATP and Mg^{2+}. This suggested that the system might be amenable to dissection. Initial experiments on centrifuged liver homogenate cells suggested that the whole process of adrenaline activated glycogen breakdown occurred in the supernatant (i.e., the *cytosol*). However, when more stringently separated cytosolic fractions were prepared, activity was lost. But when a small portion of the particulate fraction (i.e., the spun-down membrane pellet) was added back, adrenaline responsiveness returned[11].

1.3.4 Heat-stable factor – cAMP

Sutherland and co-workers then found that they could do the experiment in two stages (Figure 1.8). The purified membrane pellet was separated (from the cytosol) and incubated in buffer containing adrenaline and ATP. It could be boiled yet would still retain the ability to activate glycogenolysis when added back to the reserved cytosol. The 'boiled stuff' contained something that stimulated glycogen breakdown (and glycogen phosphorylase activation) in the cytosol. They knew that the active substance was heat-stable and therefore not a protein. They also realised it was not adrenaline or ATP, because neither could stimulate glycogen turnover in purified cytosol.

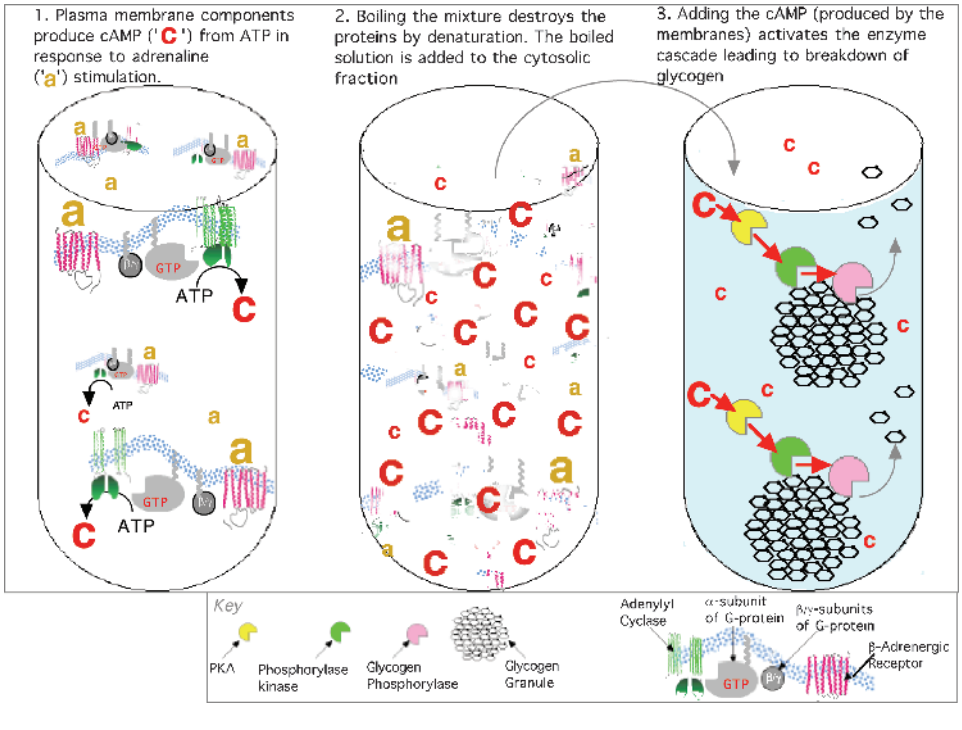

Figure 1.8 Earl Sutherland's crucial experiment

It was not possible to interpret these experiments fully at first because receptors were not understood, protein kinases were unknown and G proteins undreamt-of. However, we know now that the 'particulate' fraction contains the plasma membranes with their embedded adrenaline receptors, adenylyl cyclase and the stimulatory G protein, Gs. Incubating these membranes with adrenaline and ATP stimulates the first steps in the transduction pathway leading to the production of cAMP. The purified cytosol has no receptors, but instead contains the soluble components of the pathway: cAMP-dependent protein kinase (PKA), phosphorylase kinase (PhK), glycogen phosphorylase and the glycogen granules. Adding the cAMP (produced by the receptor-G protein-adenylyl cyclase coupling that occurred during the membrane incubation) to the cytosol activates PKA, and then PhK and finally glycogen phosphorylase itself.

The hunt was now on to find the structure of this heat-stable second messenger. It took little time to purify, crystallise, and then solve the structure of adenosine 3′, 5′-cyclic monophosphate (Figure 1.2). Unfortunately the hypothesis that this cyclic nucleotide was a universal hormonal mediator met with initial scepticism when the findings were announced.

1.3.5 The problem with rats

One serious objection (that persisted right up to the award of Sutherland's Nobel laureate) was that many labs simply could not demonstrate adrenaline-stimulated, cAMP-mediated, activation of PhK. Unfortunately, these labs were all using the most common of lab animals, rats. And they were always young males (females and older males being used primarily for breeding). It has since become clear where the problems lay: young male rat liver contains no responsive beta-type adrenaline receptors, instead adrenaline acts solely through hepatic alpha-1 receptors that stimulate PhK through calcium mobilisation alone. During their lifetimes, however, all rats do have hepatic receptors that can activate PhK via cAMP generation – female rats express both alpha and beta-adrenergic receptors, as do males that are either very young or post-mature[12] (see also Chapter 5, Section 5.7.2).

1.3.6 The discovery of hormonally regulated protein kinases – phosphorylase kinase, serine phosphorylation and Ca^{2+} – Krebs and Fischer, 1958–1968

Serendipity also played a role in the next step in unravelling how glycogen breakdown can be initiated by either hormonal (adrenaline, glucagon) or electrical (neuronal) stimulation. Strangely, filter paper had a large part to play. . .

In the 1950s, Edwin Krebs and Ed Fischer were working in the laboratories of Carl and Gerty Cori, assisting in purification of glycogen phosphorylase. The Coris were interested in the mechanism by which muscle glycogen phosphorylase *b* (the inactive form) could be converted to glycogen phosphorylase *a* (the active from) as a

result of neuronal stimulation. All that was known at the time was that supra-physiological concentrations of 5′-AMP could activate the enzyme – a finding of Krebs that remained unpublished for some time – but this allosteric modulation by AMP was not considered likely to explain activation *in vivo*. Krebs and Fischer were tasked with crystallising the *a*-form of the enzyme using a reliable purification scheme worked out by the Coris.They were unsuccessful. Their extract contained only impure *b*-form that refused to crystallise. They soon realised that they had not followed the Coris' protocol exactly: instead of 'clarifying' the muscle homogenate by filtration through filter paper, they had centrifuged it.

When they repeated the Coris' procedure exactly (with filtration through paper instead of centrifugation), they obtained active phosphorylase-*a*, which crystallised readily. A few more experiments provided two more clues. First, leaving the homogenate sitting around for too long ('ageing') prevented the conversion of *b* to *a*, even using the filtration method. Second, if the filter paper was stringently washed prior to use, the filtration method did not cause conversion even when fresh muscle homogenate was used[13].

1.3.7 Discovery of calcium as activator of phosphorylase kinase

Since the centrifugation step yielded only *b*-form, it was concluded that muscle contained predominantly inactive glycogen phosphorylase-*b*, and that the filter paper (in the Coris' protocol) had artefactually activated it. The activating contaminant (which could be washed out of the filter paper) turned out to be *calcium* and the essential component destroyed by ageing the homogenate proved to be *ATP*.

Krebs and Fischer identified a 'converting enzyme' that could be separated from glycogen phosphorylase and discovered that this enzyme, when provided with ATP and calcium, could convert phosphorylase-*b* to the *a*- form. ATP, at the same time, was converted to ADP. It was quickly concluded that this was a phosphotransferase step, glycogen phosphorylase being activated by the covalent addition of a phosphoryl group. The 'converting enzyme' was dubbed *phosphorylase kinase*, the first protein kinase discovered. They also identified the serine residue of GP whose phosphorylation led to the activation.

Phosphorylase kinase (PhK) uses ATP as a co-substrate to phosphorylate, and activate, its protein substrate GP. Further, Krebs and Fischer had established that PhK could itself exist in inactive and active forms, interconverted by loss or addition of calcium. This explained how neuronal stimulation induced glycogen breakdown in skeletal muscle – through release of calcium.

1.3.8 cAMP-dependent protein kinase

Further work in Kreb's lab established that highly purified PhK could be alternatively activated by a combination of ATP and cAMP. The activation of PhK was this time due to

its serine phosphorylation, and at first this alternative activation mechanism was thought to be due to PhK phosphorylating itself (i.e., autophosphorylation). This turned out to be incorrect. Instead a minor protein contaminant was responsible. This 'phosphorylase kinase *kinase*' proved to be a distinct protein kinase that was activated by cAMP and was capable of phosphorylating other proteins as well as PhK. It was termed 'cAMP-dependent protein kinase' and is almost universally referred to as *protein kinase A* or PKA.

1.4 The discovery of receptors and G proteins

1.4.1 Radioligand receptor assays prove receptors are discrete entities

As a philosophical concept, the 'receptor' has a long history, but in the first half of the 20th century there was no expectation that receptors might turn out to be simple proteins. Rather, the term was shorthand for a cellular 'mechanism' that produced a biological endpoint after hormonal stimulus. By the late 1960s and early 1970s, receptor binding assays were being performed with radiolabelled hormones on cells and extracts and this allowed the *Scatchard* equation and other mathematical modelling (see Chapter 2) to be used to quantify receptors directly, rather than relying upon indirect assays of downstream effects (such as phosphorylase activation and glycogenolysis). But even as late as 1973, the prominent pharmacologist Ahlquist, who developed the concept of separate alpha and beta adrenergic receptors, stated: 'To me they are an abstract concept conceived to explain observed responses of tissues produced by chemicals of various structure. . .' (see Reference 14).

1.4.2 Oestrogen receptor directly detected by radioligand binding assays – Jensen and Gorski, 1962

The first receptor to be extracted and assayed by radioligand binding assays was the oestrogen receptor in 1962. This work of Toft and Gorski followed ground-breaking research by Elwood Jensen who first used radiolabelled oestradiol to follow the fate of the hormone in rats, noting that it accumulated in target tissues (uterus and vagina) but not in non-target tissues (muscle, kidney, liver), and that it was *chemically unaltered*. This disproved the notion that oestrogens were metabolised to somehow provide energy for the biological response provoked (i.e., growth stimulation of breast cancer)[15].

Jenson's work also provided the first indications that receptors may be simple proteins. Sucrose density ultracentrifugation analysis gradients of [^{3}H]-oestradiol-bound oestrogen receptors showed a discrete '8S' band – 'S' stands for Svedberg units, an indirect measure of molecular mass, named after the Swedish chemist and Nobel Laureate, Theodor Svedberg.

1.4.3 Purification of the β-adrenergic receptor – Caron and Lefkowitz, 1976

In 1970, Lefkowitz was the first to use a radioactive ligand to label and assay a *membrane* receptor directly. The ligand was radio-iodinated adrenocorticotrophic hormone (ACTH) and the receptor was from adrenal gland membranes[16]. Iodination became a standard way of labelling peptides and proteins, whereas small ligands such as catecholamines and steroids are usually tritiated. In 1976, the β-adrenergic receptor was first purified to homogeneity and was shown to exhibit stereospecific binding to agonists and antagonists[17]. The final proof that the receptor, G protein and adenylyl cyclase were separate proteins came with the demonstration that a fully functional adrenergic-activated adenylyl cyclase could be constructed by reconstitution of the three isolated proteins into artificial phospholipid vesicles[18].

1.4.4 The discovery of G proteins. Guanine nucleotides, fluoride and aluminium – Gilman and Rodbell, 1971–1983

The magical 'contaminant' in Sutherland's ATP was re-visited after previously reliable experiments ceased to work when pure ATP analogue was used. At the same time, an important discovery was made because glass test tubes were substituted with plastic ones. . .

Alfred G. Gilman and Martin Rodbell worked independently; Gilman in Sutherland's old lab at Case Western Reserve University (by this stage run by Theodore Rall) and Rodbell at NIH, Bethesda.

Rodbell's group found the first clue that a 'transducer' may couple multiple receptors to the single effector enzyme, adenylyl cyclase (AC)[19]. Rodbell had shown that multiple hormones (including adrenaline, ACTH, TSH, LH, secretin, and glucagon) could all activate AC in fat cells. His key insight was that they were not additive in effect when applied in combinations. This argued against Sutherland's guess that each receptor had its own individual AC activity and the assumption that the receptor and AC may actually be a single entity (Figure 1.9).

1.4.5 Magnesium

Sutherland had earlier shown that the fluoride ion could stimulate AC independently of hormones[20] and Rodbell found that both ACTH and fluoride were able to stimulate AC in fat cell membranes in a MgATP-dependent manner. However, fluoride stimulation of AC exhibited Mg^{2+}-dependence with a Hill coefficient of 2 (not 1 as expected), suggesting two sites for Mg^{2+} action. One site must be AC (which used MgATP to produce cAMP), so what might the other be?

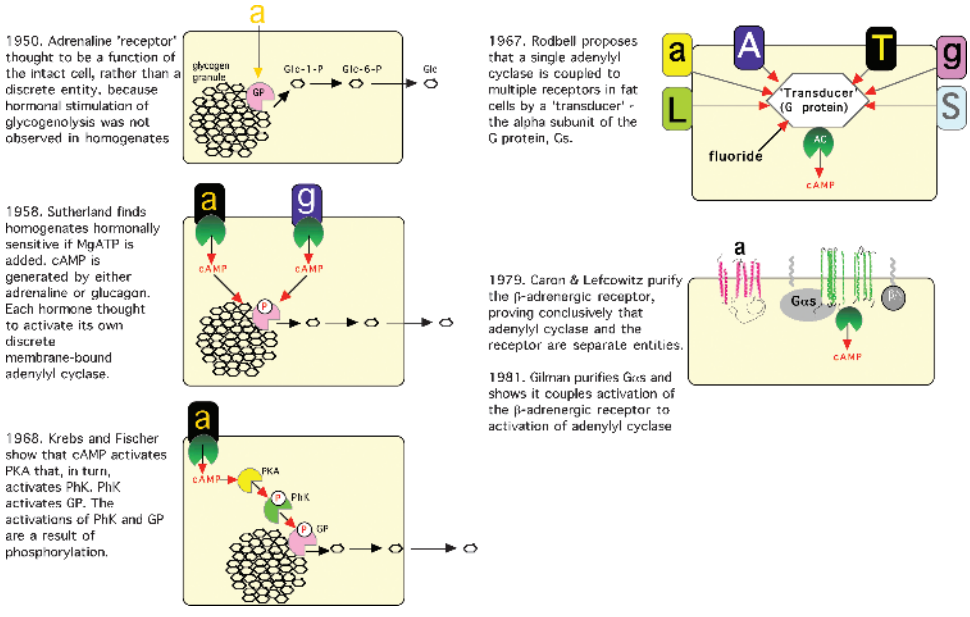

Figure 1.9 The evolution of ideas of how hormones work

1.4.6 High and low glucagon affinities

Rodbell's group found that direct binding of ^{125}I-glucagon to liver membranes did not match the kinetics of AC activation. In AC assays, glucagon raised cAMP levels within seconds of addition to liver membranes, and was quickly reversed when the hormone was removed. In binding assays, however, ^{125}I-glucagon remained bound to the membranes even after removal of free label. The label could not be displaced even with extensive washing. The problem lay in the protocols: the ^{125}I-glucagon binding assay was carried out in simple buffer, whereas the AC assay contained MgATP. Adding MgATP to the ^{125}I-glucagon assay produced a dramatic change in binding: ^{125}I-glucagon binding was easily and quickly reversed after the simple removal of ^{125}I-glucagon.

1.4.7 GTP (contaminant of ATP) lowers 7-pass receptor affinity

Rodbell knew that commercial ATP preparations were contaminated with other nucleotides. After testing a variety of candidates, he found that very low concentrations of GTP mimicked his contaminated ATP – the presence of GTP effectively lowered the affinity of the receptor for glucagon ($^{+}$GTP → fast off-rate; $^{-}$GTP → slow off-rate).

Conclusively, a pure synthetic ATP analogue (App(NH)p) did not support AC activation by glucagon unless GTP was also added[21].

Lefkowitz's group confirmed this affinity shift in adrenaline receptors and further showed that the affinity-lowering effects of GTP applied only to adrenergic receptor *agonists*; the affinity of *antagonists* was the same whether GTP was present or not[22]. It is worth noting that in both studies, GTP and GDP were equipotent in causing agonist affinity shifts. Nevertheless, such affinity-lowering effects on G protein coupled receptors are much more pronounced if non-hydrolysable GTP analogues are used, such as: guanyl-5′-imidodiphosphate (Gpp(NH)p) or guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS).

As we shall see, this affinity-lowering effect of GTP analogues towards 7-pass receptors and their agonists is a general phenomenon that came to be used to identify such G protein coupled receptors *in vitro* and to predict agonist versus antagonist behaviour *in vivo*. It is important to note that 'agonist affinity-shift' is only observable in isolated plasma membranes and is not found in whole cell receptor assays, nor in solubilised receptor preparations. Frustratingly, the mechanism behind the agonist affinity-lowering effects of guanine nucleotides is still a matter of controversy (Chapter 2).

1.4.8 GTP analogues and adenylyl cyclase activation

Artefactual AC-stimulating effects of GDP were encountered in some early studies. This proved to be due to the AC assay conditions[23], which included ATP-regenerating systems to maintain an effective level of ATP (AC substrate) against a high background of nucleoside triphosphatase activity. Unfortunately this had the unintentional side effect of converting GDP into GTP.

Significantly, there is evidence that something like this can occur in nature. G protein-bound GDP can be converted to GTP by the action of nucleoside diphosphate kinase (NDPK), an enzyme that associates with G protein β/γ subunits. Unusually, NDPK phosphorylates the β subunit on a histidine residue and this high-energy phosphate group is subsequently transferred to GDP, providing a receptor-independent means of activating G proteins. This unusual activation pathway plays a part in the aetiology of congestive heart failure[24].

The mechanism of G protein activation was clarified when adenylyl cyclase assays were performed using 5′-adenylylimidodiphosphate (App(NH)p) as a substrate instead of the ATP regenerating systems. Salomon and Rodbell clearly showed that, in these clean assay conditions, only GTP or Gpp(NH)p could support glucagon stimulation of AC activity. GDP had no effect. In the presence of Gpp(NH)p, the effects of glucagon were much longer lasting than with GTP. Further work showed that GTP activation of AC was reversible because the nucleotide is hydrolysed by the G protein, which then reverts to the inactive GDP-bound state[25]. Gpp(NH)p and GTPγS are both non-hydrolysable and so produce persistent AC-activating effects that resemble the effects of cholera toxin (see below).

1.4.9 cAMP toxicity and clonal mutants of S49 cells

The next stage in the isolation of G proteins is rather confusing, if told as it unfolded, so I intend to spare you the brain-churning interpretations that Gilman's team must have gone through.

The key tool was the lymphoma cell line, 'S49', which is killed when excess cytosolic cAMP is generated. Wild-type S49 cells contain both β-adrenergic receptors and a functional adenylyl cyclase system. Chronic adrenaline treatment thus selects mutants that do not make cAMP in response to adrenergic stimulation. Two clones were isolated that were resistant to killing by adrenaline: *cyc*$^-$ and *unc* mutants. Both cell lines express adrenergic receptors but are nevertheless resistant to adrenaline-induced cell death.

Since *cyc*$^-$ cells could not produce cAMP in response to fluoride ion, it was assumed that they simply lacked AC[26]. In a failed attempt to restore activity, AC was detergent-extracted from AC+ve cells then incubated with *cyc*$^-$ cell preparations. With hindsight, we can see that this would never have worked because AC is an integral membrane protein and cannot re-integrate into a native plasma membrane. The same can be said for the receptor. The best one can do is construct an artificial membrane vesicle around the protein using pure phospholipids.

Eventually, it was found that adrenaline-responsive AC activity could be restored to *cyc*$^-$ plasma membranes by adding extracts from wild type S49 or the B82 cell line (that was capable of cAMP production but lacked β-adrenergic receptors). B82 plasma membranes were dissolved in detergent then mildly heat-treated (37°C, 30 minutes) to destroy AC activity. When the heat-inactivated membrane extracts were incubated with *cyc*$^-$ membranes, activation in response to both adrenergic agonist and fluoride cells was restored. Whatever the restorative factor was, it could not be adenylyl cyclase, and it was obviously not β-adrenergic receptor. As it transpired, the heat-resistant factor was the alpha subunit (Gαs) of the stimulatory G protein Gs, which is the only component of the AC system missing from *cyc*$^-$ cells (it had first been thought that AC was absent).

This works because the G protein is a peripheral membrane protein, only associating through a covalently bonded fatty acid chain that (unlike an integral protein's transmembrane helix) can easily re-integrate into native membranes.

The S49 *unc* mutant is interesting from our point of view because, although able to produce cAMP when stimulated by fluoride, it is unresponsive to adrenaline, even though the cell line possesses β-adrenergic receptors. The *unc* cell line *does* express Gαs but the protein is defective because it contains a point mutation near the *C*-terminus. An arginine at Gαs position 389 (6 amino acids from *C*-terminus; number includes start methionine, see Table 9.2) is substituted with a proline. This Arg → Pro substitution prevents receptor coupling, a finding we shall come back to in later chapters.

The guanine nucleotide responsive factor, Gs, was purified to homogeneity by Gilman's group in 1980[27]. Reconstituted into phospholipid vesicles, the purified protein was able to mediate the activation of AC activity by fluoride and it was found that the protein dissociated into α- and β/γ-subunits after binding GTP (with stoichiometry of one GTP-to-one α-subunit. Incidentally, they had also unwittingly purified the

AC-inhibitory G protein, Gi, without immediately realising it (see Reference 28) – in fact, Gi-containing fractions from their gel filtration columns languished in a freezer for some time before finally being identified by labelling with pertussis toxin (discussed later).

1.4.10 Aluminium is needed for fluoride activation of G proteins

The final piece of serendipity did not reveal its full importance for another ten years. Here is an extract from Gilman's Nobel speech:

> Additional work on the mechanism of activation of Gs by fluoride provided surprises and even amusement. The effect of fluoride, observable when experiments were performed in glass test tubes . . . was lost . . . when experiments were done in plastic test tubes.
>
> Paul Sternweis . . . purified the coactivator from . . . aqueous extracts of disposable glass test tubes. A metal seemed to be involved, and neutron-activation analysis revealed Al^{3+} as the culprit.

So fluoride ion is not enough, the real inorganic stimulator of Gs-proteins is aluminium fluoride.

1.4.11 Use of bacterial toxins

At this point it is worth noting that the work of Rodbell and Gilman was paralleled and greatly assisted by the findings of many others: pre-eminently Pfeuffer and Cassel, who respectively demonstrated that GTP binding[29] and hydrolysis[30] accompanied adenylyl cyclase activation.

Gilman group's final achievement of Gs purification was by gel filtration with monitoring for AC activation in the eluted fractions, but the scheme depended partly upon labelling the Gαs subunit with a bacterial toxin excreted by *Vibrio cholerae*.

In 1978, Cassel and Pfeuffer[31], and simultaneously Gill and Meren[32], first produced evidence of a distinct guanine nucleotide binding protein by the use of cholera toxin, which (as they correctly guessed) is an ADP-ribosylating enzyme. The use of the toxin was also prompted by a number of observations including their findings that (a) cholera toxin could activate AC while at the same time inhibiting a GTPase activity downstream of adrenaline binding and (b) a GTP-binding protein was indispensable for the fluoride activation of AC[33].

Cholera toxin (CTX) is an enzyme that specifically transfers ADP-ribose from NAD^+ to the αs subunit of Gs – a reaction known as 'ADP-ribosylation' – and this was used by Cassel and Pfeuffer to radiolabel the alpha subunit of the AC-stimulating G protein. Radioactivity from the substrate ([^{32}P]-labelled NAD^+) is transferred to an arginine of Gαs by the catalytic activity of CTX. Thus labelled, it was easy to estimate the G protein's molecular weight following electrophoresis, blotting and autoradiography.

This provided a simple and reliable means to identify Gαs and, in the longer term, led to cholera and pertussis toxin (PTX) labelling being used as a generic method for discriminating between AC-stimulating, and AC-inhibiting G proteins in widely different species. Perhaps more importantly, investigations into the effects of the two toxins led to new insights into G protein mechanisms. Both toxins are exquisitely specific: CTX only labels a single arginine found in an equivalent position in all Gαs subunits (Arg201 of Gαs), whereas PTX labels a single *C*-terminal cysteine residue similarly conserved in all Gαi subunits. The effects of both modifications and their relevance to molecular details of Gα coupling mechanisms are discussed in Chapter 8.

Pertussis toxin is also an ADP-ribosylating enzyme but it is specific for the alpha subunit of the AC-inhibiting G protein, Gαi. PTX was used by Gilman's group to radio-label, and hence identify and purify, the 41 kDa AC-inhibitory G protein from the side fractions kept from their earlier Gs purifications[34].

By 1986, Lefkowitz's group had purified the alpha-2 adrenergic receptor and a consensus model of opposing adrenergic mechanisms of AC regulation had emerged.

1.4.12 The calcium signal

The ability of adrenergic agonists to stimulate PhK in young male rat liver was not fully understood until the discovery by Michael Berridge of a third type of G protein in 1984[35]. The G protein was at first referred to as 'Np', later 'Nq' and finally was given the name 'Gq'. Activation of the AC-inhibitory Gi protein can, in some instances, also lead to calcium mobilisation but this is pertussis-toxin sensitive. In the case of Gq activation, the calcium release is insensitive to both cholera and pertussis.

1.5 cAMP pathways

1.5.1 A simple mammalian signalling pathway – F-2,6-bisP as a second messenger

Fructose-2,6-bisphosphate (F-2,6-bisP) is a side-product of glycolysis that is not used for energy production, but instead acts as a second messenger. It was discovered in 1980 and was soon shown to be a widespread metabolic indicator in eukaryotes, and under hormonal control in certain mammalian tissues.

The directional flux of glycolysis versus gluconeogenesis is subject to moment-to-moment control by energy-sensing allosteric enzymes but these local controls can be over-ridden by signals (hormones) sent from remote endocrine organs.

An understanding of how the two levels of control work is contained in a classical model of metabolic pools and control points in glycolysis (Figure 1.10):

- Many enzymatic conversions are easily reversible, others (usually those requiring energy [ATP] or releasing energy [ATP]) are not.

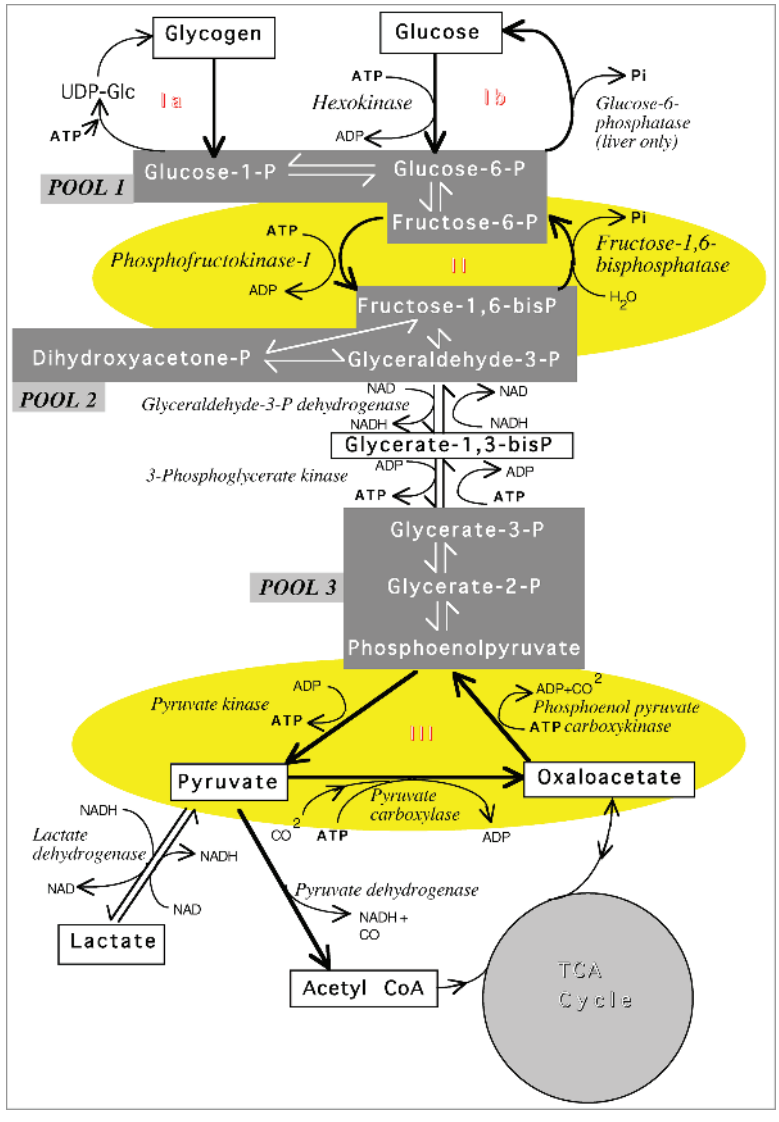

Figure 1.10 Metabolic control points in glycolysis & gluconeogenesis

- Reactions that cannot be reversed represent *control points*. This is where, for example, glycolysis uses one enzyme, but the reverse gluconeogenic step uses a different enzyme.

- Metabolites that can be interconverted by reversible reaction (using the same single enzyme and only responding to changes in substrate/product levels) are said to be in *metabolic pools*.

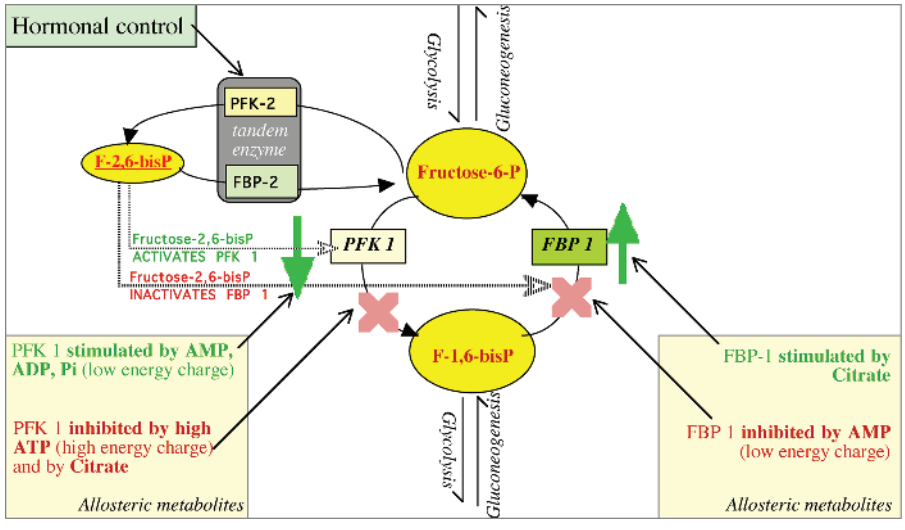

Figure 1.11 Allosteric and hormonal control of PFK-1 and FPB-1

- Control points between pools are theorised to be responsible for the overall direction of the metabolic flux.

- The important control point enzymes are *allosteric*, with active sites that can be modulated by molecules binding at non-overlapping sites ('allosteric sites') – substrate turnover may be either stimulated or inhibited (Figure 1.11).

Glycolysis should operate when energy and carbon skeleton supplies are limited – low [ATP] and [citrate]; high [ADP] and [AMP]. The pathway may be reversed, when energy supplies and carbon skeleton building blocks are high, so as to store energy in macromolecules (polysaccaharides such as glycogen, triglycerides, proteins). The 'anabolic' direction of flux is then gluconeogenic. Gluconeogenesis should only operate when energy supplies and carbon skeleton supplies are elevated – i.e., when [ADP] and [AMP] are low and [ATP] and [citrate] are high.

1.5.2 PFK-1 and FBP-1

A primary control point is 'control point II' where fructose-6-phosphate (F-6-P) is phosphorylated to fructose-1,6-bisphosphate (F-1,6-bisP) by phosphofructokinase-1 (PFK-1) or de-phosphorylated by fructose-1,6-bisphosphatase (FBP-1). Both enzymes are allosteric and respond in opposite ways to the same metabolic indicators, such as ATP, AMP and citrate – low energy charge indicators stimulate PFK-1, but inhibit FBP-1; high energy charge indicators inhibit PFK-1, but stimulate FBP-1 (Figure 1.11).

1.5.3 PFK-2/FBP-2 – a 'tandem' enzyme

A second F-6-P kinase/phosphatase system exists that controls the phosphorylation of position 2 of F-6-P. F-6-P can be 'diverted' from mainstream metabolism by being phosphorylated by phosphofructokinase-2 (PFK-2) to give fructose-2,6-bisphosphate (F-2,6-bisP), which can be de-phos-phorylated back to F-6-P by a FBP-2 activity. Whereas PFK-1 and FBP-1 are separate proteins, PFK-2 and FBP-2 activities turned out to be due to a single polypeptide, a so-called 'tandem enzyme'.

F-2,6-bisP acts as an alternative allosteric modulator of PFK-1 and FBP-1, stimulating PFK-1 and inhibiting FBP-1 (Figure 1.11). And, because the 'tandem enzyme' that produces or destroys it, is itself under hormonal control, F-2,6-bisP is effectively a second messenger in certain tissues. Its tissue-specificity is also instructive in that the same hormone/receptor combination can elicit very different signalling outcomes.

PFK-2/FBP-2 is the product of four genes in humans and there are many splice variants. The 'liver' isozyme comes as three isoforms from the same gene (*PFKFBP1*); the 'heart' isozyme is found as two isoforms from the same gene (*PFKFBP2*); the 'brain' gene (*PFKFBP3*) produces two isoforms; the testes gene (*PFKFBP4*) produces an isozyme that, unlike the others, is tissue-specific[36]. The 'liver' gene produces the ***L*** isoform in liver cells but in skeletal muscle, the same gene produces the ***M*** isoform, which differs from the ***L*** form at the *N*-terminal region. The ***L*** isoform has an *N*-terminal 32 residue regulatory region (including a PKA phosphorylation site); in the ***M*** isoform, this is replaced with an unrelated sequence lacking phosphorylation sites (Table 1.2).

1.5.4 Control of PFK-2/FBP-2 by phosphorylation – liver

The single PKA phosphorylation site of the liver ***L*** isoform is the main point of control. When stimulated by glucagon or adrenaline, the cognate hepatocyte 7-pass receptor is activated, couples with the stimulatory G protein (Gs), and causes its activation by GDP dissociation and GTP binding. The activated αs subunit then couples with AC to produce

Table 1.2 Organisation of PFK-2/FBP-2 isozyme domains

	Regulation	Catalytic	Catalytic	Regulation	Ratio of PFK2:FBP2 activities§
	PKA ↓				
Liver '*L*' form	Ser32	PFK	FBP		3:1
'*M*' form		PFK	FBP		1:5
				PKA or PKB ↓ ↓	
Heart isozyme		PFK	FBP	Ser466---Ser483	80:1
				↑ AMPK	

§Hue, L & Rider, M.H. (1987) Role of fructose 2,6-phosphate in the control of glycolysis in mammalian tissues. *Biochem. J.*, **245**: 313-324.

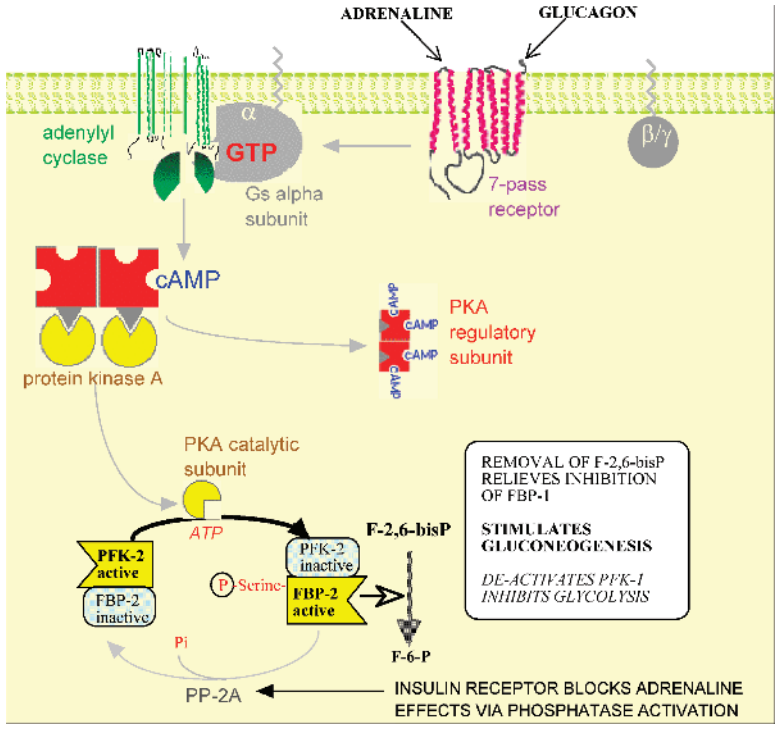

Figure 1.12 Hormonal control of glycolysis in liver by PFK-2/FBP-2 phosphorylation

cAMP. cAMP binds to the regulatory subunits of PKA, causing dissociation of the active catalytic subunits. The freed PKA phosphorylates the ***L*** tandem enzyme on Ser32 and this has two effects: (1) The PFK-2 activity is inhibited and (2) the FBP-2 activity is stimulated. The consequences of glucagon / adrenaline stimulation is that the second messenger F-2,6-bisP disappears, PFK-1 is inactivated by its loss and the inhibition of FBP-1 is relieved. This effectively switches the pathway from glycolysis to gluconeogenesis. This is just one of the ways in which the liver responds to the body's need for glucose export in starvation or stress (Figure 1.12). In the liver, insulin blocks adrenaline effects by activating a phosphdiesterase, destroying cAMP and potentially allowing de-phosphorylation to occur (Chapter 9, Section 9.8). The tandem enzyme activity ratio can be reversed by de-phosphorylation of Ser32 by protein phosphatase-2A.

1.5.5 Control of PFK-2/FBP-2 by phosphorylation – heart

The heart isozyme is controlled by phosphorylations of its *C*-terminal regulatory domain and its hormonal control presents a most unusual example of insulin and adrenaline signals producing the same outcome. The heart is highly adaptable in terms of energy requirements and will happily use low energy fuels like lipids or lactate, but

when glucose is plentiful after a meal or when fight-or-flight stimulus requires extra energy, the heart switches to glycolysis. Similarly, in anaerobic conditions the heart also must switch to glycolysis because the TCA cycle shuts down.

Downstream of adrenaline, PKA phosphorylates Ser466 and Ser483 of the heart tandem enzyme and this has the effect of activating the PFK-2 activity with little effect upon the FBP-2 activity, which is already low[36]. The net effect of PKA activation, then, is an increase in glycolytic flux. Interestingly, insulin has the same effect of increasing glycolysis when glucose is abundant. Insulin activates a serine/threonine kinase (related to PKA) called PKB (see Chapter 9). PKB (or another insulin-regulated kinase) phosphorylates the same two sites on the heart PFK-2/FBP-2 enzyme with the same outcome of increasing glucose usage via glycolysis (Figure 1.13). Recently, it has been found that this phosphorylation by PKB leads to the phospho-PFK-2/FBP-2 being bound by a 14-3-3 protein, an event that contributes to the activation[37]. 14-3-3 proteins are phospho-serine binding proteins (see Chapter 3, Section 3.7.1).

The 'Pasteur effect' describes the shift from energy production by the TCA cycle to glycolysis in anaerobic conditions. Underlying this effect is an increase in 5′-AMP, which stimulates a 5′-AMP-dependent protein kinase (AMPK). AMPK can also phosphorylate the heart isozyme on Ser466, again with the same outcome: glycolysis is stimulated.

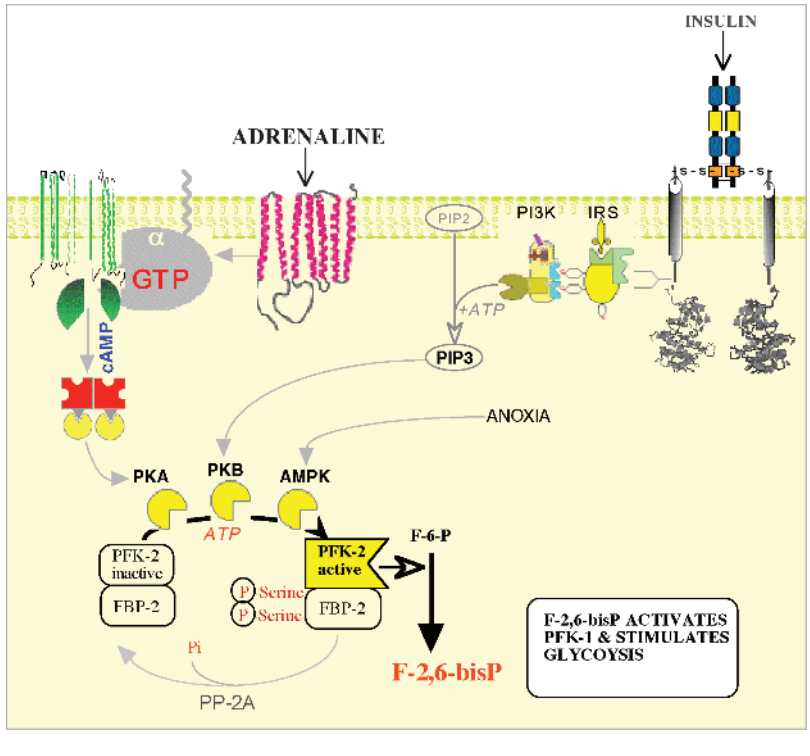

Figure 1.13 Hormonal control of glycolysis in heart

1.5.6 F-2,6-bisP in tumours

Many types of cancer cells produce large amounts of lactate even in aerobic conditions – the 'Warburg effect'. PFK-2 isozymes are often highly active in cancer cells and levels of F-2,6-bisP are high. It is theorised that this may contribute to the dominance of anaerobic glycolysis in cancer cells[38], although a more direct explanation is that at least some cancers have a defective glycerol phosphate shuttle and are forced to use lactate (rather than the mitochondrion) to regenerate NAD^+.

1.6 cAMP: ancient hunger signal – primitive signalling in amoebazoans and prokaryotes

1.6.1 Slime moulds

Being part-time multicellular animals, slime moulds appear to be positioned somewhere between unicellular eukaryotes and metazoans in terms of complexity; as such, social amoebae are often termed *amoebazoans*.

The most striking feature of slime moulds is their ability to circumvent starvation by swarming together to form a slug that can migrate and differentiate into a fruiting body. This process is activated and guided by a single molecule but, since the *Dictyostelium* genome has been completely deciphered, this seemingly simple system has proved to be much more complex than expected. The following is a summary.

When food runs out, individual *Dictyostelium* amoebae in the soil begin to secrete bursts of cAMP every six minutes. cAMP acts as a *chemoattractant*. Nearby cells sense the stimulus via cell surface 7-pass cAMP receptors (cAR) receptors and begin to move up the concentration gradient towards the source of cAMP. Such directional migration towards a chemoattractant is known as *chemotaxis*. At the same time, these responding amoebae begin to secrete cAMP themselves, setting up concentric waves of migration towards a central collecting point. cAMP is produced by an *adenylyl cyclase*. The on-off six minute pulses are achieved by a negative feedback mechanism that desensitises the receptor by destroying the extracellular cAMP long enough for the cell's cAMP receptor to recover, sense the cAMP concentration gradient, and then begin secreting more of its own cAMP. The destruction of cAMP is caused by an *extracellular phosphodiesterase* (PDE) (Figure 1.14).

The culmination of all this chemotaxis signalling is the formation of multicellular motile 'slug' made up of as many as 100,000 amoebae. The slug migrates towards light and once it has found a suitable position on the surface it begins to *differentiate* into stalk cells and spore cells. The spore cells become desiccated and eventually are dispersed by the wind, to germinate into individual amoebae if conditions are right, or remain dormant until conditions improve. The stalk cells, however, are sacrificed in the process.

The most remarkable fact is that the whole process of sporulation (including swarming, differentiation and eventual germination) is controlled by a single molecule, cAMP, and key types of pathway components shared with humans.

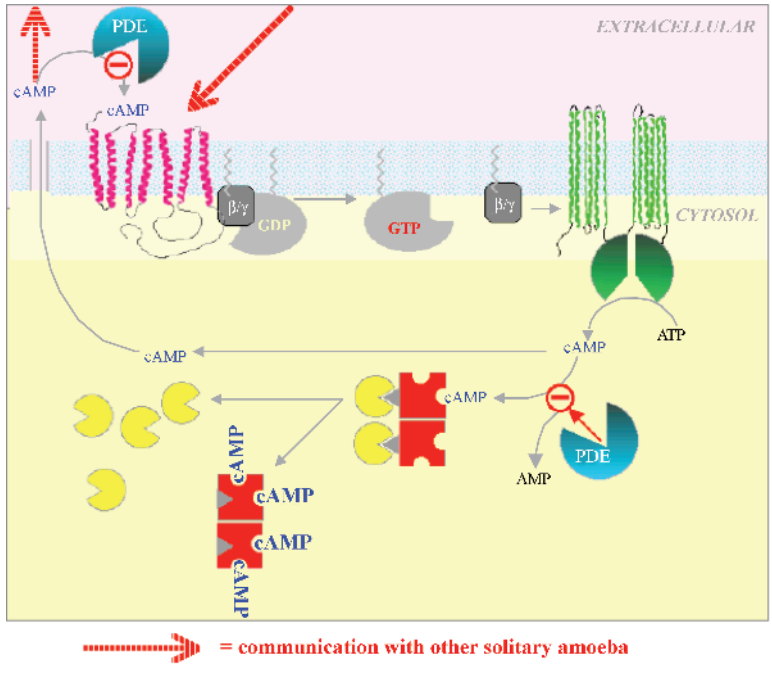

Figure 1.14 cAMP signal transduction in slime mould

The *Dictyostelium* genome is now fully sequenced and although the genes for the components of the chemotaxis/differentiation pathways are known, few of the gene products' activities have been biochemically characterised.

Dictyostelium has six chromosomes with an estimated 12,500 genes. There are at least 55 genes for 7-pass G protein-coupled-receptors (GPCRs) and these can be grouped into those resembling mammalian GPCR families 2, 3 and 5 (see Chapter 5, Section 5.1) and the unrelated family of cAMP receptors (cAR1, cAR2, cAR3 and cAR4) and cAMP receptor-like GPCRs (CRLs) that are unique to slime moulds[39]. The genome contains 14 heterotrimeric G protein alpha subunits, but only two Gβ and a single Gγ gene are present[40]. The G protein alpha subunits (Gα1–Gα12) are all most closely related to the mammalian Gαi family[41]. In addition, there are at least 40 monomeric G proteins, including members of Ras/Rap, Rac, Rab, Ran and Arf families; the effector pathways of Ras (MAP kinase or Erk) are also present, as are the regulators of Ras (GEFs and GAPs)[42].

The genome codes for three adenylyl cyclases that are involved in *Dictyostelium's* development programme and their activities are opposed by the activities of both extracellular and cytosolic PDE enzymes.

A major difference in slime mould GPCR signalling, compared to metazoans, is the pre-eminence of G protein β/γ subunits in activation of *Dictyostelium* adenylyl cyclase. Whereas *all* mammalian adenylyl cyclases are activated by Gαs subunits[43], in slime mould the Gα subunits have no such effect on the enzyme.

The membrane-bound, Gβ-coupled adenylyl cyclase A (ACA) of *Dictyostelium* is homologous with mammalian adenylyl cyclases, sharing the same structure of *N*-terminal 6-pass transmembrane domain, followed by a cytosolic catalytic domain, separated by a second 6-pass transmembrane domain from a second, *C*-terminal, catalytic domain that makes up the other half of the split catalytic site. ACA controls the chemotaxis and aggregation stage of multicellular development. A second cyclase (ACG) is G protein-independent and controls spore development. It consists of a single transmembrane domain and a single catalytic domain and is controlled by osmolarity[7]. The third adenylyl cyclase is cytosolic and is responsible for correct spore development. This enzyme is referred to as either 'ACB'[44] or 'ACR'[45].

ACA is coupled to cAR1 via the heterotrimeric G protein Gα2β/γ but, unlike mammalian adenylyl cyclases, ACA is activated solely by the β subunit (Figure 1.14). This appears reminiscent of type II and IV mammalian adenylyl cyclases, which are also activated by β/γ. However, activation of mammalian adenylyl cyclases by β/γ is conditional, being dependent upon the presence of an activated Gαs subunit, which must also bind simultaneously to AC II at an alternative site to that occupied by β/γ[43] (see Chapter 8, Section 8.6).

Their developmental programme involves not only metazoan-like signalling molecules that are absent in unicellular forms (SH2 domains, for example), but it also retains ancient signal systems absent in metazoans. A good example of the latter, prokaryote-like system is the His/Asp phosphorelay used by *Dictyostelium* to control RegA, one of its phosphodiesterases[46]. Like RegA, ACR also contains a prokaryotic response regulator domain[46].

1.6.2 cAMP and *E. Coli*

In 1965, Makman and Sutherland[47] made the surprising discovery that cAMP was present in the cytoplasm of the enteric bacterium, *E. coli*, and that the concentration of the cyclic nucleotide increased as growth slowed down when glucose was depleted or removed from the medium. Under these conditions, the bacteria are forced to utilise less favoured fuel sources like lactose, which are more costly to metabolise and consequently less energy efficient. 'Catabolite repression' was, at the time, a relatively well-understood phenomenon due to the work of Jacob and Monod who discovered that for *E. coli* to metabolise lactose, a whole new set of genes needed to be transcribed – the *Lac* operon. The *Lac* operon contains three 'structural' genes encoding β-galactosidase, thiogalactoside transacetylase, and a transporter (galactoside permease). It was first thought that a catabolite of glucose was responsible for repressing the transduction of these genes, and that when glucose was used up, the 'catabolite' disappeared and the 'repression' was lifted. It later transpired that this was the opposite of what was happening, but the name 'catabolite repression' stuck and survives today.

In the absence of glucose, *E. coli* needs to find alternatives. It does this by sensing the background turnover of other sugars present in the environment. Lactose, for example, is a substrate for one of the *Lac* operon enzymes (β-galactosidase) that is present in very

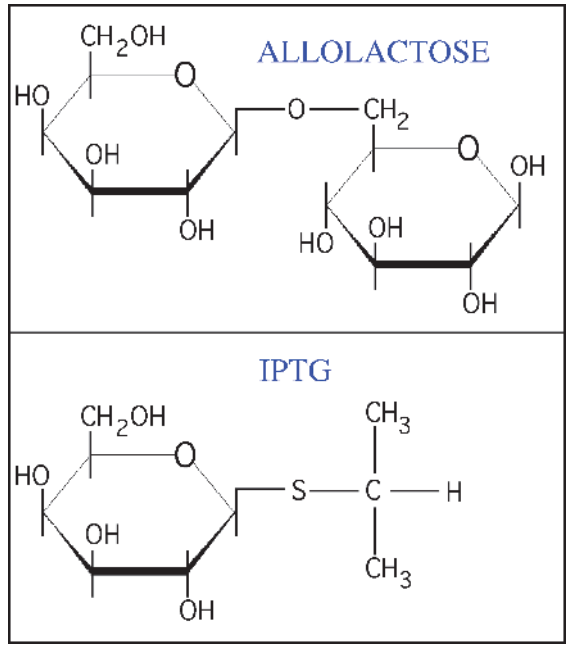

Figure 1.15 Lac operon inducers

low amounts even in the presence of glucose. β-Galactosidase normally hydrolyses lactose to galactose plus glucose, but is also capable of catalysing the production of small amounts of *allolactose*. Allolactose binds to the *Lac* repressor protein, altering its conformation such that it can no longer bind to the DNA of the operator site upstream of the structural genes of the operon. De-binding of the *Lac* repressor protein allows RNA polymerase to bind weakly, but fully productive binding is only achieved if cAMP is also present *at high levels*. That is because cAMP binds to the transcription factor CAP (catabolite activator protein). CAP is a primordial cAMP receptor that bears a remarkable structural resemblance to the main vertebrate cAMP receptor, the R-subunits of PKA (see Chapter 4). It is worth noting that a structural analogue of allolactose is in wide use today: *isopropylthiogalactoside* (IPTG) (see Figure 1.15) is used as a non-hydrolysable inducer of the *Lac* operon in artificial protein expression systems.

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