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G-protein-coupled receptors: past, present and future

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The G-protein-coupled receptor (GPCR) family represents the largest and most versatile group of cell surface receptors. Drugs active at these receptors have therapeutic actions across a wide range of human diseases ranging from allergic rhinitis to pain, hypertension and schizophrenia. This review provides a brief historical overview of the properties and signalling characteristics of this important family of receptors.
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Abbreviations: CGRP, calcitonin-gene-related peptide; CK1α, casein kinase 1α; CRLR, calcitonin-receptor-like receptor; DADL, [D-Ala^2,D-Leu^5]enkephalin; DCI, dicholorisoprenaline; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; ICI 174864, [*N*, *N*′-diallyl-Tyr^1, Aib^{2,3}]Leu^{5}enkephalin; PKA, protein kinase A; PSD-95, postsynaptic density-95; RAMP, receptor-activity-modifying protein; SPAP, secreted placental alkaline phosphatase; TM, transmembrane

Introduction

The G-protein-coupled receptor (GPCR) family represents the largest and most versatile group of cell surface receptors, which can detect a diverse array of chemical signals in a highly selective way and then transduce the signal from these ligand–receptor interactions into intracellular responses. Drugs active at these receptors have therapeutic actions across a wide range of human diseases ranging from allergic rhinitis to pain, hypertension and schizophrenia. The human genome project has identified more than 800 different GPCR genes and yet the majority of GPCR drugs in current clinical practice (which represent more than 30% of all drugs) exert their actions on only approximately 30 of them (Wise *et al*., 2004). There are therefore enormous opportunities for further drug discovery in the field of GPCRs. In addition, our increasing knowledge of the complexity of the signalling mechanisms activated by GPCRs, the cellular organisation of the proteins with which they interact and the mechanisms by which they are regulated, provide new challenges to our basic understanding of the pharmacology of this important class of cell surface receptors. In this review, I have attempted to provide a brief historical overview of this important field of research.

The early conceptual framework and the ability to monitor affinity, efficacy and signal amplification

The idea that drugs bind to specific sites or receptors on cell surfaces stemmed originally from the work of Paul Ehrlich (1854–1915) in Germany who studied the interaction of dyes with biological structures (reviewed by Drews, 2004). The term 'receptive substance' was first coined in Cambridge by John Langley (1852–1925) and further developed by Sir Henry Dale. However, the major legacy left by the early pioneers of pharmacology (such as Clark, Gaddum, Stephenson and Schild who all worked in the U.K.) to the field of GPCR research and pharmacology in general is the development of the concepts of affinity and efficacy (see also Rang, this issue). They also pioneered the quantitative methods by which the properties of agonists, partial agonists and antagonists can be evaluated from the measurement of functional responses in isolated tissues (Arunlakshana & Schild, 1949; Stephenson, 1956). Their application of the law of mass action to classical concentration–response data and the development of rigorous approaches to monitor the competitive nature of antagonist–receptor interactions to yield quantitative estimates of antagonist affinity has stood the test of time and is still the mainstay by which pharmacologists study receptor-stimulated responses today (albeit increasingly in recombinant cell lines rather than in isolated tissues). These scientists reasoned that provided the chemical nature of the drug molecules and receptors did not change, then the affinity of an antagonist for a given receptor should be the same whatever tissue was used and whichever agonist was used to stimulate it. The application of the methods of Gaddum, Schild and Stephenson to the measurement of the affinity constants of antagonists and partial agonists is illustrated in Figure 1, with data obtained from measurement of gene transcription responses to a GPCR in transfected cells. If you consider the Gaddum equation (Figure 1), then it will be clear to you that the concentration of antagonist, which requires a doubling of the concentration of agonist in order to obtain the same size response, will be the antagonist dissociation constant. The logarithm of the reciprocal of this concentration is classically represented by the pharmacological symbol pA_2. pA_2 is also the title of the British Pharmacological Society's online journal and an excellent overview of the use of this method of analysis is provided by Richard Barlow in the second issue of this E-Journal (Barlow, 2003) who had synthesised many of the partial agonists used by Stephenson in Edinburgh to develop his efficacy theory.

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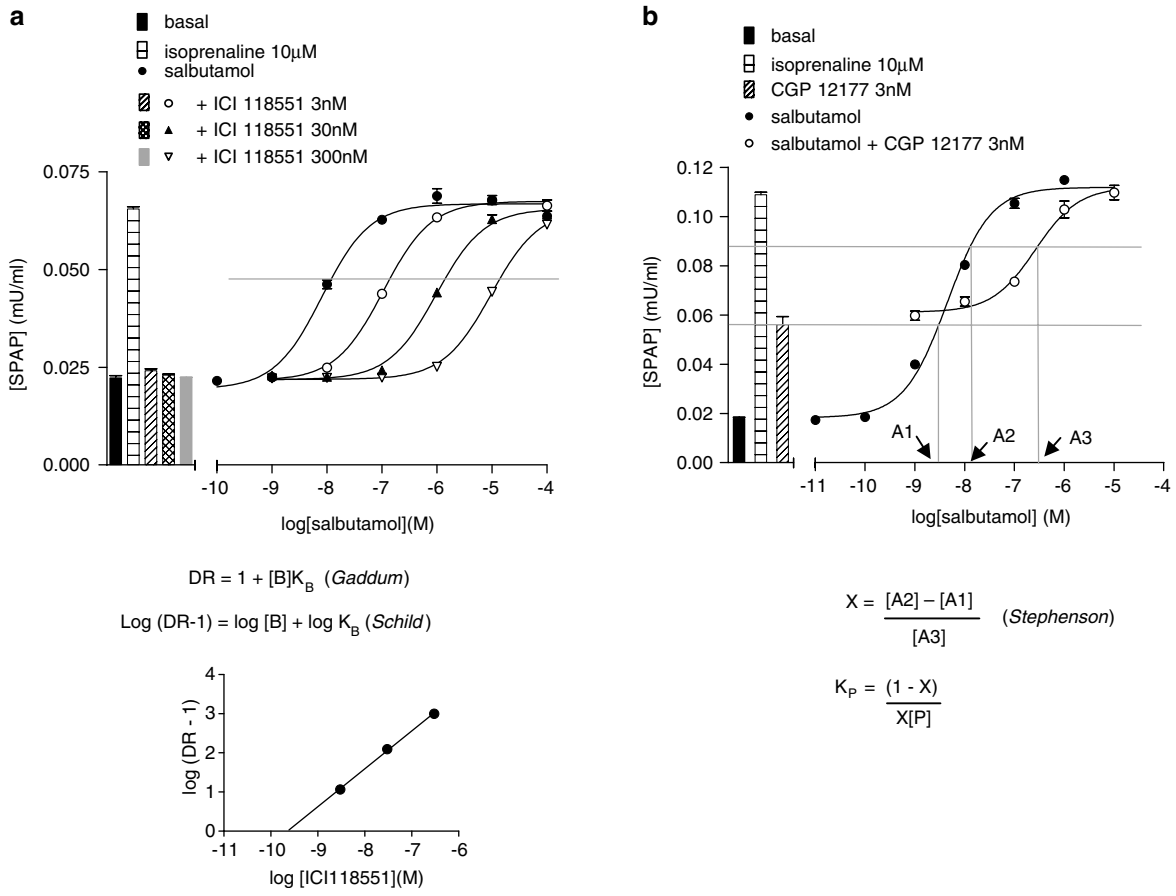

Figure 1 Examples of the use of the equations developed by Gaddum, Schild and Stephenson to determine the affinity constants of antagonists and partial agonists for GPCRs. (a) Antagonism by ICI 118551 of the CRE-reporter gene response (SPAP, secreted placental alkaline phosphatase) to salbutamol in CHO cells expressing the human β_2-adrenoceptor. For each concentration of the antagonist ICI 118551, the ratio (DR, dose ratio) of salbutamol concentrations required to produce the same sized response (indicated by the grey line) in the presence and absence of the antagonist is determined. The antagonist affinity constant (K_B) can then be determined either directly from the Gaddum equation or from a Schild plot of log (DR-1) against the log of the antagonist concentration ([B]). Data (unpublished observations) were kindly provided by Dr Jillian Baker. (b) Antagonism by CGP 12177 of the CRE-reporter gene response to salbutamol in CHO cells expressing the human β_2-adrenoceptor. In this case, 3 nM CGP 12177 alone produces a partial agonist response. If the concentrations of salbutamol required to produce the same sized response in the presence (A3) and absence (A2) of CGP 12177 are determined along with the concentration of salbutamol alone that produces the same response as 3 nM CGP 12177 alone (A1), then the affinity constant of CGP 12177 (K_P) can be determined as shown. In the original analysis by Stephenson (1956), he denoted x as the proportion of receptors occupied by partial agonist. The term shown as X therefore equates to $(1-x)$ described in Stephenson's original formula. Data taken from Baker *et al*. (2002).

The concept of agonist efficacy, an awareness of spare receptors and the consequences of signal amplification within different tissues and cells have been (and still are) vital to the study of GPCRs. Receptor reserve can best be described as the extent to which agonist log concentration–response curves are shifted to the left in a parallel manner as a consequence of signal amplification. In a poorly coupled system, an agonist concentration–response curve may overlay the position predicted by measurement of ligand binding (see below), whereas an increase in receptor concentration in a common cell background can produce a leftward shift in agonist concentration–response curves. Compounds that appear as weak partial agonists in low expressing systems can therefore behave as full agonists in higher expressing systems. Furthermore, measurement of agonist responses at different levels of an intracellular cascade can lead to curves which gradually move to the left as you move down the signalling cascade as a consequence of increased amplification.

The chemical toolkit – selective agonists and antagonists

The ability to study the detailed pharmacological properties of GPCRs by definition relies on the availability of compounds that can selectively stimulate and antagonise responses mediated by a particular GCPR. This is best illustrated by the difficulties experienced today in the study of orphan receptors. These are GPCRs identified within the human genome as proteins that possess the classic seven transmembrane spanning domains, but for which no natural or synthetic ligands have been identified. Although current estimates vary, there are probably still more than 150 human orphan receptors.

The power of collaboration between chemistry and pharmacology is immense and the success of this alliance has underpinned astonishing successes in the development of powerful therapeutic drugs for a wide range of human

diseases. Pharmacology has always been a multidisciplinary science and it has been the biochemists, chemists, pharmacists, physiologists and clinicians coming from other disciplines who have made it what it is today. In my view, pharmacology is a way of thinking and designing experiments. Its exponents compare the activities of natural and synthetic compounds on a biological target (usually a GPCR) and use quantitative measurements to gain insight into mechanisms of action. However, without the collaboration between medicinal chemistry and the analytical approach of pharmacology, the advances in drug discovery would have been minimal.

This synergy between chemistry and pharmacology in the development of novel drugs for GPCRs is perhaps best illustrated by the work of Sir James Black and his co-workers at ICI (Alderley Park, Macclesfield, U.K.) and SKF (Welwyn Garden City, U.K.) in the development of β-blockers and histamine H_2-receptor antagonists (Black *et al*., 1965; 1972). In each case, the starting point was a well-recognised clinical problem (angina pectoris or gastric and duodenal ulcers). The approach was to build analytical pharmacological models and to evaluate in a quantitative fashion the impact of modification of the natural hormone or neurotransmitter. In both cases, the lead to a receptor selective antagonist was the identification of a partial agonist. However, this is not a trivial task because of the tissue-dependent nature of partial agonism and the impact of signal amplification on the manifestation of full or partial agonist activity of low-efficacy molecules. As Sir James observed in his Nobel lecture (Black, 1993), ''As analytical pharmacologists, what we are allowed to see of a new molecule's properties is totally dependent on the techniques of bioassay we use. The prismatic qualities of the assay distort our view in obscure ways and degrees''. His solution was to develop new assays. This led to the identity of dichloroisoprenaline as a partial agonist for the β-adrenoceptor and the development of propranolol as a β-adrenoceptor antagonist. In much the same way, Robin Ganellin, Michael Parsons and Sir James identified guanylhistamine as a partial histamine H_2-receptor agonist and then developed the H_2-receptor antagonist cimetidine (see also Parsons & Ganellin, this issue).

Radioligand binding studies – a first venture into molecular characterisation

The development of radioligand-binding studies in the late 1960s and early 1970s provided the means by which new insights into the molecular identity and properties of GPCRs were achieved. The first study of this type was by William Paton & Humphrey Rang (1965) in Oxford (U.K.) who studied the binding of ^{3}H-atropine to muscarinic receptors in strips of the longitudinal muscle of guinea-pig ileum (see also Rang, this issue). This was followed a few years later by Arnold Burgen, Robin Hiley, Alan Cuthbert and Michael Young in Cambridge (U.K.) who investigated receptor-selective binding of the irreversible ligand ^{3}H-propylbenzilylcholine mustard to muscarinic receptors in chick amnion muscle and guinea-pig ileal smooth muscle (Burgen *et al*., 1974 and references therin). During the early part of this decade, similar advances were made with binding studies using radiolabelled agonist and antagonists for a large number of GPCRs. My own involvement in ligand-binding studies began when I undertook my PhD studies in the laboratory of Michael Young in Cambridge (Hill *et al*., 1977).

Much of the research effort for a number of receptor systems in the early 1970s related to the growing realisation that the receptor structures responsible for stimulating cyclic AMP formation involved separate proteins for agonist recognition (receptor), cyclic AMP synthesis (adenylyl cyclase) and the transduction of information from the receptor to adenylyl cylase (heterotrimeric G-protein). This work led to the concept of GPCRs and the vital role of G-proteins in signal transduction processes. This largely arose from the considerable efforts of the groups of Martin Rodbell (North Carolina, U.S.A.) and Alfred Gilman (Dallas, Texas, U.S.A.) and is well summarised in their respective Nobel lectures (Gilman, 1997; Rodbell, 1997; see also Milligan & Kostenis, this issue).

A clear feature that was generally observed in the binding of ligands to a particular GPCR (although at the time most of them were not considered as GPCRs) was the fact that the displacement of radiolabelled antagonist binding to cell membranes in basic buffer systems by agonists was characterised by flat curves (low Hill coefficients) that appeared to contain high- and low-affinity components. In contrast, the displacement of radiolabelled antagonist binding by antagonists was generally consistent with a simple mass–action relationship involving only one binding site. Nigel Birdsall and co-workers at the National Institute of Medical Research (London, U.K.) produced the most comprehensive data for this in the case of muscarinic receptors in brain membranes using both ^{3}H-antagonists and ^{3}H-agonists and with Philip Strange also applied the approach to neuroblastomal cells (Birdsall *et al*., 1978; Strange *et al*., 1978). In the case of G_s-coupled receptors it was also observed that, in the presence of GTP, the agonist displacement curves in membrane preparations were shifted to higher agonist concentrations and the Hill slopes increased towards unity (becoming consistent with a single binding site). This led Andre DeLean and Robert Lefkowitz (Durham, U.S.A.) to propose the 'ternary complex model' involving high (G-protein-coupled) and low (not coupled) agonist affinity states of the β-adrenoceptor (DeLean *et al*., 1980). The idea at the time was that antagonists did not discriminate between G-protein-coupled and uncoupled receptors (but see under inverse agonism below).

Radioligand-binding studies and the need for parallel functional studies

As the number of radioligands available to study receptor binding increased exponentially in the 1970s and 1980s, it became clear that there was a need to ensure that the binding detected represented binding to the target receptor and not to some other nonspecific site with an avid affinity for the radioligand. Indeed, the literature contains nice examples of mistaken receptor identities ranging from glass-fibre filters to metabolic enzymes. The confirmation of the correct target was usually best achieved by comparing the binding affinities of antagonists determined from radioligand studies in membrane preparations with those determined from classical analysis of functional responses using the techniques developed by Gaddum and Schild.

The biggest problems, however, were in comparing binding and function in brain tissues. Henry Bourne and Paul Insel

(both San Francisco, U.S.A.) had made considerable progress in studying receptor-mediated cyclic AMP accumulation in leukocytes and S49 lumphoma cells (Bourne *et al*., 1971; Insel & Kennedy, 1978). John Daly (NIH, Bethesda, U.S.A.) and Stefan Nahorski (Leicester, U.K.) also succeeded in measuring cyclic AMP formation in cerebral cortical slices in response to adenosine and β-adrenoceptor stimulation (Schultz & Daly, 1973; Nahorski, 1977). John Daly's group was also responsible for the discovery that forskolin from the roots of *Coleus forskolii* was a direct activator of adenylyl cyclase (Seamon *et al*., 1981). He was subsequently able to show that GPCR-mediated inhibition of forskolin-stimulated adenylyl cyclase via G_i-proteins could also be demonstrated in brain membrane preparations (Seamon & Daly, 1981). However, at the time, there were no readily amenable biochemical assays for G_q-coupled receptors. Michael Hanley and Leslie Iversen had some success in Cambridge with muscarinic receptors and cyclic GMP accumulation in brain slices (Hanley & Iversen, 1978) but it was not readily amenable to other receptors. In our own case with the H_1-receptor, we had to result to measuring a crosstalk interaction involving amplification by histamine of adenosine-stimulated cyclic AMP accumulation in guinea-pig cerebral cortical slices (Hill *et al*., 1981). However, this assay 'need' at the time did result in a long-standing interest in the mechanisms underlying the crosstalk between different GPCRs (Selbie & Hill, 1998).

The big breakthrough for G_q-coupled receptors came from the observation by Peter Downes in Cambridge, in collaboration with Michael Berridge and Michael Hanley, that lithium ions could amplify the ability of agonists such as carbachol, histamine, 5-HT and substance P to stimulate ^{3}H-inositol phosphate accumulation in brain slices (Berridge *et al*., 1982; see also Nahorski, this issue). Lithium inhibited the metabolism of ^{3}H-inositol phosphates to ^{3}H-myo-inositol and thus allowed the accumulation of ^{3}H-inositol phosphates to be measured in tissues prelabelled with ^{3}H-myo-inositol. They then designed a simple assay based on ion-exchange chromatography that allowed simple measurements of inositol phospholipid metabolism. Subsequently, Stefan Nahorski (Leicester, U.K.) and others were able to apply this technique to a range of different G_q-coupled receptor systems (Brown *et al*., 1984). In 1985, Roger Tsien (Berkeley, California) synthesised a family of highly fluorescent calcium indicator dyes (Quin-2 and subsequently fura-2, fluo-3, and fluo-4), which allowed the measurement of intracellular calcium changes in living cells in response to GPCRs and made the real-time study of G_q-coupled receptor function possible in cultured cells (Grynkiewicz *et al*., 1985).

Receptor purification, molecular cloning and mutagenesis

A major consequence of the ability to identify specifically GPCRs using radioligands was that it provided the means by which the receptors could be purified and followed through the purification process in a highly selective way. The major effort in this activity came from the groups of Robert Lefkowitz and Marc Caron at Duke University (Durham, U.S.A.) who succeeded in purifying to homogeneity first the β_2-adrenoceptor and then the α_{2A} and α_{1B} adrenoceptors (reviewed in Lefkowitz, 2004). As a consequence, one of the most important breakthroughs in the field of GPCRs was achieved in 1986 with the cloning of the hamster β_2-adrenoceptor from a genomic library constructed by Brian Kobilka in the laboratories of Robert Lefkowitz (Durham, U.S.A.) and Merck (New Jersey, U.S.A.) (Dixon *et al*., 1986). This revealed for the first time that the β_2-adrenoceptor shared the predicted seven transmembrane (7TM) structure of the visual pigment rhodopsin. This knowledge and the suspected homology with already-cloned members of the GPCR family led to a rapid expansion of the number of cloned receptors (Lefkowitz, 2004). Furthermore, following the sequencing of the human genome, we now know that this receptor family is very large and as mentioned earlier there are probably still 150 or so nonsensory GPCRs without a known ligand. What is also interesting is that GPCRs have now been identified which have very unexpected ligands including GPCRs for nicotinic acid, bile acids, fatty acids, calcium ions and trace amines (Wise *et al*., 2004). We should also not forget the considerable number of olfactory receptors that are also GPCRs.

The impact of the cloning of the first GPCR has been immense and the availability of the cDNA for GPCRs has revolutionised the means by which their pharmacology and function can be studied. By transfecting single receptor subtypes into appropriate host cells, receptors can now be studied in isolation without the complications of similar receptor subtypes being also present in cells. This has allowed detailed studies of their molecular properties and of their interaction with signalling partners. Site-directed mutagenesis has identified the binding sites for ligands, which in the case of biogenic amine GPCRs is deep within the transmembrane helices. It has also identified major differences between the different GPCR families and provided important information on the residues that are important for coupling to G-proteins. The key residues that are phosphorylated in response to second messenger and GPCR kinases have also been determined.

The availability of the cDNA for GPCRs and other proteins also provided the means by which GPCRs could be tagged to allow them to be visualised in real time. For example, the addition of a short nucleotide sequence to the extracellular amino terminus of a GPCR, which encodes for an epitope for a commercially available antibody (e.g. myc or haemaglutinin), allows the location of that receptor to be monitored using immunohistochemical or live cell imaging techniques. Similarly, the production of a GPCR chimeric protein constituting a GPCR with the full coding region of green fluorescent protein (GFP) attached to its intracellular C-terminus allows the location of the receptor to be monitored in real time (Figure 2). Graeme Milligan in Glasgow (U.K.) has taken this one step further and produced chimeric proteins involving G-protein α-subunits fused to the C-terminus of different GPCRs in order to study agonist-mediated regulation of G-protein and receptor palmitoylation (Stevens *et al*., 2001; see also Milligan & Kostenis, this issue).

Constitutive receptor activity and inverse agonism

One of the surprising findings that came from the early work utilising chimeric receptors was the discovery by Susanna Cotecchia in the Lefkowitz laboratory that replacing just four

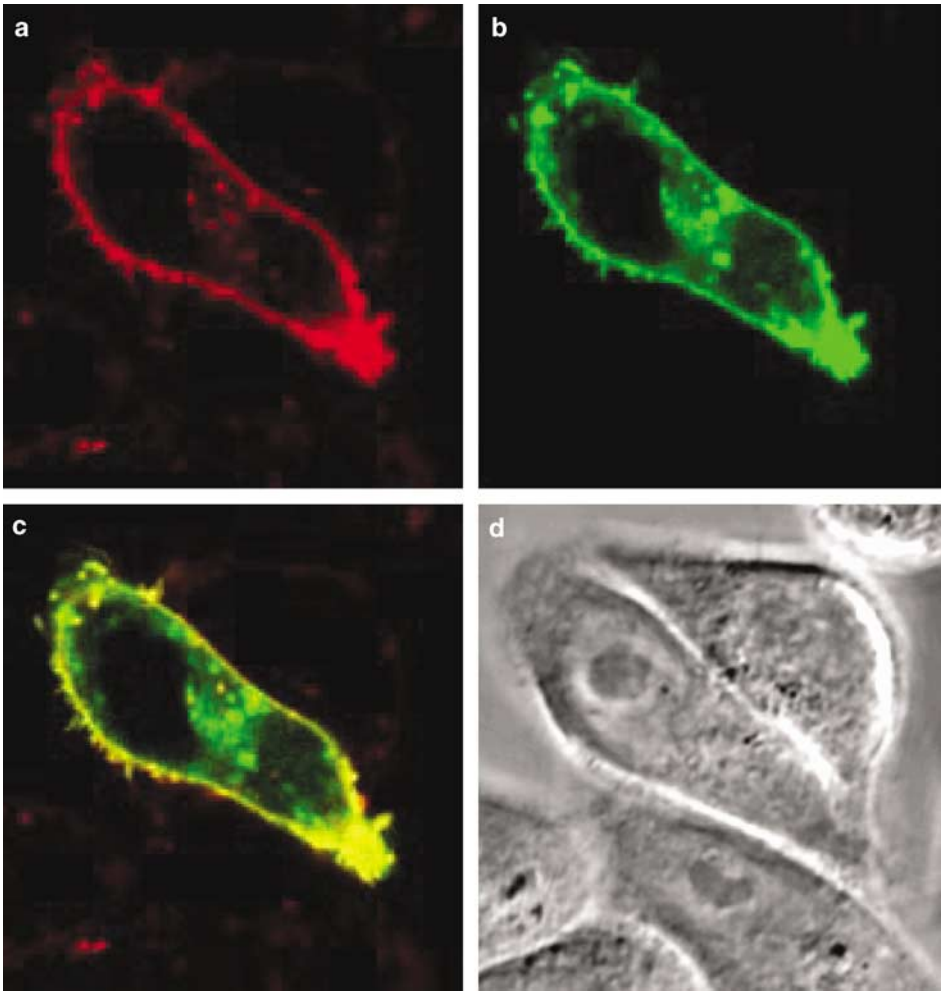

Figure 2 Binding of BODIPY-TMR-CGP12177 to CHO cells expressing the human β_2-GFP adrenoceptor. (a) Binding of 50 nM BODIPY-TMR-CGP 12177 (red channel) to the same cells as in (b) which shows the location of the GFP-tagged β_2-adrenoceptor (green channel). (c) An overlay of the two images (a, b) with colocalised pixels shown in yellow. (d) Phase contrast image of these cells (Baker J.G. & Hill, S.J. unpublished observations). The image was used as the poster for the British Pharmacological Society's 1st James Black Conference 'Activation of cell surface receptors: new insights into ligand-gated ion channels and G-protein-coupled receptors', Cambridge, 2002.

amino acids in the third cytoplasmic loop of α_{1B}-adrenoceptor with the corresponding residues from the β_2-adrenoceptor generated a constitutively active mutant receptor (Lefkowitz *et al*., 1993; Lefkowitz, 2004). The mutant α_{1B}-adrenoceptor was now able to generate an inositol phosphate accumulation in the absence of added agonist. Furthermore, the reciprocal substitutions in the β_2-adrenoceptor also produced a constitutively active mutant form of the β_2-adrenoceptor, which in this case stimulated agonist-independent stimulation of cyclic AMP accumulation.

The possibility for receptors to exist in an active (R*) or inactive (R) form first arose in order to explain why the binding of certain antagonists of G_i-coupled muscarinic, D_2-dopamine and adenosine A_1-receptors was modulated by guanine nucleotides and sodium ions (which also destabilise receptor–G-protein complexes) in a reciprocal fashion to that observed with agonists. For example, Philip Strange (Reading, U.K.) has shown that the binding of the 5-HT_{1A} agonist ligand [^{3}H]8-OH-DAT is inhibited by GTP, but that for the antagonist ligand [^{3}H]spiperone is increased (Sundaram *et al*., 1993). This led Andre DeLean and co-workers to propose an extended ternary complex model which suggested that certain antagonists may be able to promote the dissociation of the receptor from G-proteins and hence produce an inverse agonist effect (Wreggett & De Lean, 1984). This hypothesis was tested by Costa & Herz (1989) working in the Max-Planck Institute in Martinsried (Germany) using NG108-15 cells endogenously expressing the δ opioid receptor. In this classic study, they investigated the effect of opioid receptor agonists and antagonists on high-affinity GTPase activity in membranes prepared from NG108-15 cells. Gα-subunits possess inherent GTPase activity and as a consequence of increased GTP binding in the presence of activated receptor there is a subsequent increase in GTPase activity. Tommaso Costa and Albert Herz showed that 'basal' GTPase activity could be increased by replacing NaCl in the buffer with KCl (which increases the proportion of R*G complexes). They also showed that agonists could increase GTPase activity and some antagonists could markedly inhibit GTPase activity. Furthermore, these novel 'inverse agonist' effects of certain 'antago-

nists' were more noticeable at low sodium concentrations where partial inverse agonist effects could also be detected (Figure 3). Finally, they identified a neutral antagonist (MR 2266) which could competitively antagonise both the agonist effects of DADL and the inverse agonist effects of ICI 174864 (Figure 3). Thus, Tommaso Costa and Albert Herz firmly established the concept of inverse agonism.

Following the discovery of constitutively active mutants and the fact that constitutive receptor activity could also be generated (particularly for G_s-coupled receptors) by over-expression of the receptor, inverse agonists for a wide range of receptors were discovered. Many 'antagonists' in common clinical practice including β-blockers such as propranolol (Baker *et al*., 2003 and references therin) and H_2-receptor antagonists such as cimetidine and ranitidine (Smit *et al*., 1996) were subsequently shown to be inverse agonists. Indeed, with a better appreciation of the fact that a complete range of efficacies (from full agonist to full inverse agonist) is possible, it is clear that completely neutral antagonists are likely to be a rarity. Furthermore, in keeping with Sir James Black's theme to be mindful of what an assay can tell you, it is clear that the manifestation of an agonist or inverse agonist effect may well depend on the response being measured. For example, Jillian Baker (Nottingham, U.K.), Graciela Pineyro (Montreal, Canada) and co-workers have shown that propranolol can be both an inverse agonist (cyclic AMP accumulation) and a partial agonist (MAP kinase activation) in the same cell system depending on the signalling pathway that is monitored (Azzi *et al*., 2003; Baker *et al*., 2003).

Inverse agonist effects have also been observed *in vivo*. The first was undertaken by Richard Bond and Robert Lefkowitz using transgenic mice with myocardial overexpression of the β_2-adrenoceptor (Milano *et al*., 1994). However, perhaps the most intriguing has come from the work of Jean-Michel Arrang and Jean-Charles Schwartz in Paris on the histamine H_3-receptor. This receptor naturally contains within the C-terminal region of its third intracellular loop the residues that are normally mutated into a GPCR in order to make it constitutively active (Morisset *et al*., 2000). This raised the possibility that the native H_3-receptor (which is an autoreceptor that normally negatively regulates the release of histamine from histaminergic nerve terminals) may be constitutively active under normal physiological situations (see also Parsons & Ganellin, this issue). Jean-Charles Schwartz and co-workers were able to show that an inverse H_3-agonist was able to stimulate histamine release from rodent brain slices and synaptosomes and to elicit increases in brain tele-methyl-histamine levels in mice receiving inverse agonists orally. In all cases, the inverse agonist effects were reversed by the neutral H_3-receptor antagonist proxyfan (Morisset *et al*., 2000).

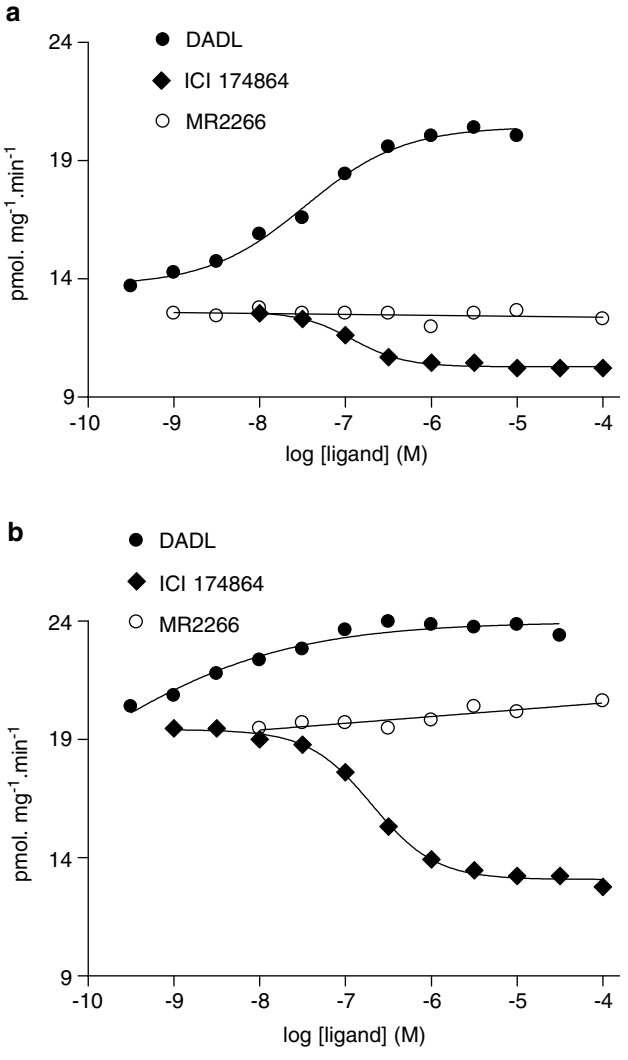

Figure 3 Agonist and inverse agonist effects of opioid ligands on GTPase activity in membranes of NG108-15 cells. High-affinity GTPase activity was measured in medium containing (a) 150 mM NaCl or (b) KCl. Data were taken from Costa & Herz, (1989). In the absence of NaCl (b), there is increased constitutive activity and the inverse agonist effects of ICI 174864 are more noticeable. MR 2266 was also able to competitively antagonise both the agonist effects of DADL and the inverse agonist effects of ICI 174864 (Costa & Herz, 1989). It therefore behaves as a neutral antagonist.

Protean agonism, agonist trafficking and the conformational cafeteria

The concept and prediction that certain agonists could reverse their effects under particular situations was first suggested by Terry Kenakin from GSK (North Carolina, U.S.A.) (reviewed in Kenakin, 2001). These agonists were termed protean after Proteus, the Greek god who could change shape and appearance at will. The hypothesis was that some agonists may produce an active receptor conformation that had a lower efficacy than the spontaneously formed conformation (in the absence of agonist) that was responsible for constitutive receptor activity (Kenakin, 2001). As a consequence, in a constitutively active system where there is a significant proportion of spontaneously active receptors (R_{spon}*) activation by a protean agonist will lead to a lower efficacy active form of the receptor (R*) and this would manifest itself as inverse agonist activity (i.e. reducing activity from R_{spon}* to R*). In a quiescent system (i.e. no constitutive activity), the ligand would produce activation (agonist effects) by virtue of changing the predominant resting form of the receptor R to R*. Thus, in a quiescent system, the protean ligand acts as an agonist and in a constitutively active system the ligand is an inverse agonist. Some experimental evidence for this has been provided by the work of Peter Chidiac and Michel Bouvier (Montreal, Canada) who have shown that dicholorisoprena-

line (DCI; the partial agonist first identified by Sir James Black, see above) is a positive partial agonist for β_2-adrenoceptors transfected into sf9 cells (reviewed in Kenakin, 2001). However, following isoprenaline-stimulated desensitization, DCI produced inverse agonism. Similar observations have been noted by the same group when cyclic AMP measurements were compared from intact sf9 cells expressing the β_2-adrenoceptor and membranes obtained from the same cells. In the latter case, the receptors are constitutively active (by virtue of lower GTP levels) and DCI (as well as labetelol and pindolol) switch from being partial agonists in intact cells to inverse agonists in membrane preparations (Kenakin, 2001). Protean agonism has also been noted for proxyfan *in vitro* and *in vivo* at the histamine H_3-receptor (which appears to be constitutively active under physiological conditions; Gbahou *et al*., 2003).

The concept of protean agonism relies on the assumption that different active states of GPCRs exist. Furthermore, it has also been noted that the relative efficacies of certain ligands are different when different responses are measured within the same cell (Berg *et al*., 1998). With our increased realisation that GPCRs can couple to more than one type of G-protein, it is possible that different agonist-induced conformations of receptors exist that have different efficacies for coupling to particular G-proteins or signalling pathways. One of the first demonstrations of this was by William Clarke (San Antonio, Texas, U.S.A.) who showed that the 5-HT_{2C} receptor can couple to two different pathways in CHO cells (inositol phosphate accumulation and arachidonic acid release) and exhibit a markedly different rank order of efficacies for each response (Berg *et al*., 1998). The experimental data were first presented by William Clarke at the Winter meeting of the British Pharmacological Society in 1996 at which Paul Leff (Loughborough, U.K.) completely independently presented a theoretical model for a GPCR existing in two active states with different preferences for particular G-proteins. The final manuscript was a direct consequence of the stimulating discussions that were initiated at that meeting. This type of ligand behaviour has been variously termed 'agonist-trafficking', 'functional selectivity' and 'stimulus trafficking'. It is likely that receptors can spontaneously adopt a variety of different conformations and that particular ligands can stabilise a specific spectrum of conformations which then lead to a set range of biological effects. Kenakin has coined the phrase 'conformational cafeteria' to describe how ligands may enter receptor space and choose their specific diet of conformations (Kenakin, 2002).

The need to consider both allosteric and orthosteric ligand-binding sites

The idea that different ligands may stabilise different conformations of a GPCR or interact with different residues within the receptor sequence also raises the possibility that GPCRs may, just like enzymes, be regulated in an allosteric fashion. Thus, a drug may bind to a site (allosteric site) that is different from the orthosteric site to which the endogenous ligand binds. In this way, an allosteric modulator may increase or decrease the binding and efficacy of the endogenous agonist. Nigel Birdsall (London, U.K.) and Arthur Christopoulos (Melbourne, Australia) have developed many of the theoretical frameworks for analysing such interactions (reviewed in Christopoulos & Kenakin, 2002; Soudijn *et al*., 2004). Examples include PD 81723, an allosteric enhancer at the adenosine A_1-receptor (Soudijn *et al*., 2004), and thiochrome, which increases acetylcholine binding to the M_4 muscarinic receptor (Lazereno *et al*., 2004).

Receptor oligomerisation and accessory proteins

It has been well established for many years that tyrosine kinase receptors function as dimers. Evidence for the concept that GPCRs may also be able to form dimers or other oligomeric species began to accumulate from the studies of Roberto Maggio and Jurgen Wess at NIH (Bethesda, U.S.A.) in which truncated GPCRs (containing transmembrane (TM) regions 1–5) were coexpressed with gene fragments encoding the C-terminal regions of the receptor (containing TM 6–7) and functional receptor activity was reinstated. This approach was further refined when α_2/M_3 and M_3/α_2 chimeras were generated in which TM 6 and 7 were exchanged between the α_{2C}-adrenoceptor and M_3-muscarinic receptors. It was only when the two chimeras were cotransfected into Cos-7 cells that functional responses and ligand binding could be generated (Maggio *et al*., 1993). Michel Bouvier, Terry Hebert and co-workers (Montreal, Canada) also used epitope-tagged receptors and Western blotting techniques to monitor homodimerisation of the β_2-adrenoceptor and to identify a peptide sequence corresponding to TM6 that could disrupt receptor dimerisation and reduce stimulatation of adenylyl cyclase (Hebert *et al*., 1996). These observations raise interesting questions regarding the stoichiometry of the receptor–G-protein interaction, that is, does a receptor dimer interact with one or two heterotrimeric G-proteins? Some insight into this is provided by recent studies on the leukotriene B_4 BLT_1-receptor which suggests that a GPCR dimer engages only one heterotrimeric G-protein in a pentameric signalling unit (Baneres & Parello, 2003).

Perhaps the most clearcut example of a receptor heterodimer that is essential for functional activity, however, comes from the interaction between the $GABA_BR1$ and $GABA_BR2$ gene products (Marshall *et al*., 1999; see also Bowery & Smart, this issue). Fiona Marshall and co-workers at GSK in Stevenage (U.K.) showed, at the same time as groups from Synaptic (U.S.A.) and Novartis (Basel, Switzlerland) in the same issue of Nature, that $GABA_BR1$ is normally poorly expressed on the cell surface, but following expression of $GABA_BR2$ there is a marked expression of heterodimers on the cell surface. Immediately following the discovery that $GABA_B$ receptors exist as heterodimers, Jordan & Devi (1999; New York, U.S.A.) reported heterodimerisation between κ and δ opioid receptors that resulted in new pharmacology quite distinct from the pharmacology of the individual component receptors (see also Corbett *et al*., this issue). More recently, fluorescence and bioluminescence resonance energy studies have provided convincing evidence that both homodimers and heterodimers exist in living cells (Bulenger *et al*., 2005). However, perhaps the most visually impressive evidence for dimerisation has come from the atomic force microscopy images of rhodopsin homodimers in native retinal disks (Fotiadis *et al*., 2003) (Figure 4).

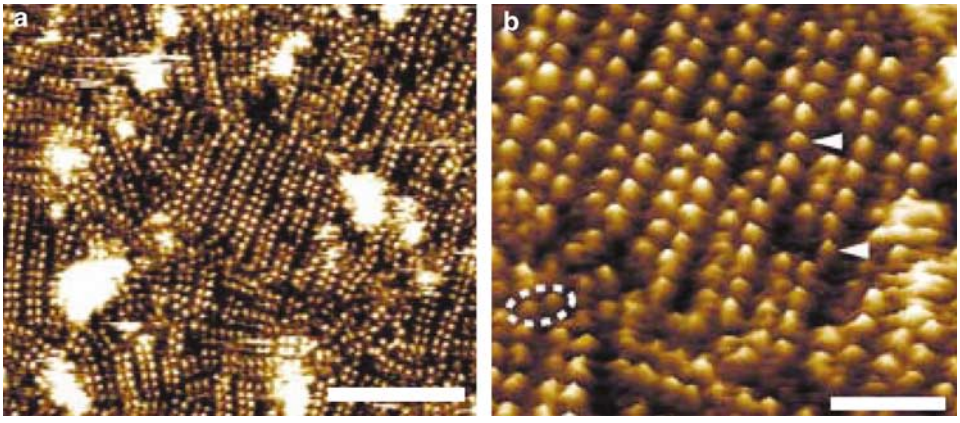

Figure 4 Organisation and topography of the cytoplasmic surface of rhodopsin. (a) Topograph obtained using atomic-force microscopy showing paracrystalline arrangement of rhodopsin dimers in a native disk membrane. (b) Magnification of a region of the topograph shown in (a) showing rows of rhodopsin dimers. Individual dimers (dashed elipse) and monomers (arrow heads) can also be observed. Scale bars (a) 50 nm and (b) 15 nm. Reproduced with permission from Fotiadis *et al*. (2003).

In addition to its role in receptor activation, evidence is accumulating that homodimerisation and heterodimerisation of GPCRs occurs early in the biosynthetic process at the level of the endoplasmic reticulum and may be an obligatory step for cell surface expression (Bulenger *et al*., 2005). However, it is not only other GPCRs that can fulfil this role and receptor-activity-modifying proteins (RAMPs) represent the first example of a single membrane spanning protein that can transport GPCRs to the cell surface and modify their pharmacology (see also Brain & Cox, this issue). These were first discovered by Stephen Foord and his co-workers in the Receptor Systems and Cell Biology groups at GSK (Stevenage, U.K.), while investigating receptors for the calcitonin family of peptides (McLatchie *et al*., 1998). The calcitonin-receptor-like receptor (CRLR) had been cloned some time previously but it had proven difficult to identify the endogenous ligand because the expressed receptor did not appear to have the pharmacology of a calcitonin-gene-related peptide (CGRP) or an adrenomedullin receptor. The GSK group finally discovered that an accessory protein was involved when they attempted to clone the gene for the CGRP receptor by an expression cloning strategy in *Xenopus* oocytes. CGRP-mediated responses were observed when the cDNA encoding a 148-amino-acid protein (RAMP1) from a cDNA library was expressed. Functional CGRP-receptor activity was restored because CRLR required RAMP1 for functional expression and that *Xenopus* oocytes already possessed an endogenous CRLR. The group then went on to show that the nature of the RAMP could determine the pharmacology of the resulting receptor: RAMP1 presents the receptor at the cell surface as a CGRP receptor, while RAMP2 transports the receptor to the cell surface as an adrenomedullin receptor.

Receptor phosphorylation, desensitisation, internalisation and recycling

It has been clear for many years that the continued binding of an agonist to a receptor does not cause a continuous response, but instead, the GPCR adapts to the presence of that stimulus and becomes desensitised (Carman & Benovic, 1998; Kahout & Lefkowitz, 2003). The β_2-adrenoceptor is the most thoroughly investigated member of the GPCR family in this respect and it was the early work by Jeffrey Benovic working in the Lefkowitz laboratory (Durham, U.S.A.) that identified the importance of receptor phosphorylation. Desensitisation of the β_2-adrenoceptor can be mediated either by a mechanism that depends on agonist occupancy (homologous desensitisation) or by a mechanism that involves the activation of protein kinase A (PKA; heterologous desensitisation). PKA leads to phosphorylation of the β_2-adrenoceptor but only causes a partial uncoupling of the receptor from its G-protein. This phosphorylation can be produced by low agonist occupancy of the receptor, since only small increases in cyclic AMP are required to fully activate PKA. In contrast, homologous desensitisation requires high agonist occupancy by a strong agonist and involves the recruitment of G-protein-coupled receptor kinases (GRKs) to the receptor (Carman & Benovic, 1998; Kahout and Lefkowitz 2003). This leads to phosphorylation of serine residues within the C-terminal tail of the β_2-adrenoceptor and the subsequent binding of β-arrestins. Binding of β-arrestin causes a disruption of the receptor–G_s-protein interaction and prevents further signalling from the receptor via the G_s-protein. β-Arrestin also acts as an adaptor coupling the receptor to clathrin-coated pits and targets the receptor for subsequent internalisation to endosomes (Luttrell & Lefkowitz, 2002). The receptor is then dephosphorylated and either recycled to the plasma membrane or targeted for degradation following ubiquitination (Shenoy *et al*., 2001). Jennifer Koenig and Michael Edwardson (Cambridge, U.K.) have undertaken an extensive mathematical analysis of this process to reveal the extent and rate of intracellular receptor movement (Koenig & Edwardson, 1997).

Evidence is also accumulating, however, that certain GPCRs can be internalised via lipid rafts and caveolae (Self *et al*., 2005 and references therein). In many cases (including our own for the H_1-receptor), the mechanisms responsible for this still remain to be elucidated. However, in the case of the β_1-adrenoceptor, phosphorylation of the receptor by PKA is responsible for directing internalisation *via* caveolae (Rapacciuolo *et al*., 2003). In addition to GRKs and second messenger-regulated protein kinases (e.g. PKA), Andrew Tobin working in Leicester (U.K.) with Stefan Nahorski discovered that casein kinase 1α (CK1α) is responsible, at least

in part, for the cellular phosphorylation of muscarinic M_3 receptors (Tobin *et al*., 1997). He has also shown that M_3 receptor phosphorylation by CK1α contributes to the activation of the MAP kinase pathway by muscarinic agonists (Budd *et al*., 2001). This raises the prospect that there may be a number of other enzymes still to be discovered that can phosphorylate GPCRs in a stimulus-dependent manner.

Coupling to other signalling proteins and the importance of location

β-Arrestin2 has recently also been shown to act as a scaffold for activation of MAP kinase cascades by G-protein-coupled receptors and can function to retain activated MAP kinase enzymes within the cytosol rather than allowing them to translocate to the nucleus where they would normally stimulate gene transcription (Luttrell *et al*., 2001; Seta *et al*., 2002; Wei *et al*., 2003). Futhermore, it is now clear that this interaction between GPCRs and β-arrestins can activate intracellular signalling *via* MAP kinase independently of the involvement of heterotrimeric G-proteins (Seta *et al*., 2002; Azzi *et al*., 2003; Wei *et al*., 2003). As a consequence, GPCRs may have a wider signalling function than can be ascribed to their ability to bind to heterotrimeric G-proteins. GPCRs can associate with a number of other scaffolding, chaperone and signalling proteins, which act to deliver them to a particular signalling complex and to orchestrate the resulting responses. These proteins include (a) RAMPs (see above); (b) Homer, which binds to metabotropic receptors and plays a key role in trafficking them to the cell surface; (c) postsynaptic density-95 (PSD-95), which binds to the C-terminus of the β_1-adrenoceptor and stabilises the receptor at the cell surface; (d) caveolin-1, which has a key role in the formation of caveolae and (e) A-kinase anchoring protein 79 (AKAP 79; Fraser *et al*., 2000; Gines *et al*., 2001; Tan *et al*., 2004). In this latter case, John Scott (Portland, U.S.A.) has provided exciting evidence for the presence of a signalling complex comprising the β_2-adrenoceptor, PKA, protein kinase C, protein phosphatase PP2B and AKAP 79 (Fraser *et al*., 2000). Finally, Hall *et al*. (1998) have shown that the β_2-adrenoceptor can interact directly with the Na^+/H^+ exchanger regulatory factor and control Na^+/H^+ exchange.

The future

The increased evidence of compartmentalisation of GPCR-mediated signalling as a consequence of scaffolding in larger molecular complexes and their association with lipid rafts and caveolae (Ostrom & Insel, 2004 and references therein) raises the prospect of microdomain-specific signalling and pharmacology. Our increased knowledge of receptor location must also be matched to the accumulating evidence for agonist-specific signalling and the importance of both temporal and spatial aspects of intracellular signalling. As a consequence, there is the real prospect that the molecular pharmacology of a given ligand–receptor interaction may differ between microdomains within a single cell. The challenge for the future is to develop the technology to be able to study receptors in these domains. Fluorescent ligands and GFP-tagged GPCRs have been developed for this purpose (Figure 2) and recent advances include our own application of fluorescence correlation spectroscopic techniques (Briddon *et al*., 2004) to real-time measurement of ligand binding to GPCRs in membrane microdomains and the FRET-based approaches developed by Martin Lohse (Wurzburg, Germany) to monitor real-time changes in receptor conformation (Hoffman *et al*., 2005). Classical pharmacology has in the past provided the essential means by which quantitative studies of ligand affinity and efficacy can be measured using indirect means. The challenge for the future will be to apply and adapt the same rigorous approaches, in collaboration with medicinal chemists and molecular biologists, to interrogate ligand–receptor interactions at the single molecular level in real time and in living cells.

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References

ARUNLAKSHANA, O. & SCHILD, H.O. (1949). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.,* **14,** 48–58.

AZZI, M., CHAREST, P.G., ANGERS, S., ROUSSEAU, G., KOHOUT, T., BOUVIER, M. & PINEYRO, G. (2003). Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 11406–11411.

BAKER, J.G., HALL, I.P. & HILL, S.J. (2002). Pharmacological characterization of CGP 12177 at the human β_2-adrenoceptor. *Br. J. Pharmacol.,* **137,** 400–408.

BAKER, J.G., HALL, I.P. & HILL, S.J. (2003). Agonist and inverse agonist actions of β-blockers at the human β_2-adrenoceptor provide evidence for agonist-directed signalling. *Mol. Pharmacol.,* **64,** 1357–1369.

BANERES, J.L. & PARELLO, J. (2003). Structure-based analysis of GPCR function: evidence for a novel pentameric assembly between dimeric leukotriene B4 receptor BLT1 and the G-protein. *J. Med. Biol.,* **329,** 815–829.

BARLOW, R.B. (2003). The slopes of concentration–effect (dose–response) curves or Clark, Gaddum, Schild and pA_2. PA_2 online. *E-J. Br. Pharmacol. Soc.*, http://www.pa2online.org/Vol1Issue2D-Barlow.html.

BERG, K.A., MAAYANI, S., GOLDFARB, J., SCARAMELLINI, C., LEFF, P. & CLARKE, W.P. (1998). Effect pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol. Pharmacol.,* **54,** 94–104.

BERRIDGE, M.J., DOWNES, C.P. & HANLEY, M.R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.,* **206,** 587–595.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1978). The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.,* **14,** 723–736.

BLACK, J.W. (1993). Drugs from emasculated hormones: the principles of synoptic antagonism. In *Nobel Lectures, Physiology or Medicine 1981–1990,* Frangsmyr J & Linsten J (eds). Singapore: World Scientific Publishing Co..

BLACK, J.W., DUNCAN, W.A., DURANT, C.J., GANELLIN, C.R. & PARSONS, E.M. (1972). Definition and antagonism of histamine H2-receptors. *Nature,* **236,** 385–390.

BLACK, J.W., DUNCAN, W.A. & SHANKS, R.G. (1965). Comparison of some properties of pronethalol and propranolol. *Br. J. Pharmacol.,* **25,** 577–591.

BOURNE, H.R., MELMON, K.L. & LICHTENSTEIN, L.M. (1971). Histamine augments leukocyte adenosine 3′,5′-monophosphate and blocks antigenic histamine release. *Science,* **173,** 743–745.

BRIDDON, S.J., MIDDLETON, R.J., CORDEAUX, Y., FLAVIN, F.M., WEINSTEIN, J.A., GEORGE, M.W., KELLAM, B. & HILL, S.J. (2004). Quantitative analysis of the formation and diffusion of A_1–adenosine receptor–antagonist complexes in single living cells. *Proc. Natl. Acad. Sci. U.S.A.,* **101,** 4673–4678.

BROWN, E., KENDALL, D.A. & NAHORSKI, S.R. (1984). Inositol phospholipids hydrolysis in rat cerebral cortical slices: I. Receptor characteristics. *J. Neurochem.,* **42,** 1379–1387.

BUDD, D.C., WILLARS, G.B., MCDONALD, J.E. & TOBIN, A.B. (2001). Phosphorylation of the G_q/11-coupled M_3-muscarinic receptor is involved in receptor activation of the ERK-1/2 mitogen-activated protein kinase pathway. *J. Biol. Chem.,* **276,** 4581–4587.

BULENGER, S., MARULLO, S. & BOUVIER, M. (2005). Emerging role of homo- and heterodimerization in G protein-coupled receptor biosynthesis and maturation. *Trends. Pharmacol. Sci.,* **26,** 131–137.

BURGEN, A.S.V., HILEY, C.R. & YOUNG, J.M. (1974). The binding of [^{3}H]-propylbenzilylcholine mustard by longitudinal muscle strips from guinea-pig small intestine. *Br. J. Pharmacol.,* **50,** 145–151.

CARMAN, C.V. & BENOVIC, J.L. (1998). G-protein-coupled receptors: turn-ons and turn-offs. *Curr. Opin. Neuro. Biol,* **8,** 335–344.

CHRISTOPOULOS, A. & KENAKIN, T. (2002). G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.,* **54,** 323–374.

COSTA, T. & HERZ, A. (1989). Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding protein. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 7321–7325.

DELEAN, A., STADEL, J.M. & LEFKOWITZ, R.J. (1980). A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.,* **255,** 7108–7117.

DIXON, R.A.F., KOBILKA, B.K., STRADER, D.J., BENOVIC, J.L., DOHLMAN, H.G., FRIELLE, T., BOLANOWSKI, M.A., BENNETT, C.D., RANDS, E., DIEHL, R.E., MUMFORM, R.A., SLATER, E.E., SIGAL, I.S., CARON, M.G., LEFKOWITZ, R.J. & STRADER, C.D. (1986). Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. *Nature,* **321,** 75–79.

DREWS, J. (2004). Paul Ehrlich: Magister mundi. *Nat. Rev. Drug Disc.,* **3,** 797–801.

FOTIADIS, D., LIANG, Y., FILIPEK, S., SAPERSTEIN, D.A., ENGEL, A. & PALCZEWSKI, K. (2003). Rhodopsin dimers in native disc membranes. *Nature,* **421,** 127–128.

FRASER, J.D., CONG, M., KIM, J., ROLLINS, E.N., DAAKA, Y., LEFKOWITZ, R.J. & SCOTT, J.D. (2000). Assembly of an A kinase-anchoring protein-β_2-adrenergic receptor complex facilitates receptor phosphorylation and signalling. *Curr. Biol.,* **10,** 409–412.

GBAHOU, F., ROULEAU, A., MORISSET, S., PARMENTIER, R., CROCHET, S., LIN, J.S., LIGNEAU, X., TARDIVEL-LACOMBE, J., STARK, H., SCHUNACK, W., GANELLIN, C.R., SCHWARTZ, J.C. & ARRANG, J.M. (2003). Protean agonism at histamine H_3 receptors *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 11086–11091.

GILMAN, A.G. (1997). G-proteins and regulation of adenylyl cyclase. In *Nobel Lectures, Physiology or Medicine 1991–1995,* Ringertz (ed). Singapore: World Scientific Publishing Co..

GINES, S., CIRUELA, F., BURGUENO, J., CASADO, V., CANELA, E.I., MALLOL, J., LLUIS, C. & FRANCO, R. (2001). Involvement of caveolin in ligand-induced recruitment and internalization of A_1 adenosine receptor and adenosine deaminase in an epithelial cell line. *Mol. Pharmacol.,* **59,** 1314–1323.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HALL, R.A., PREMONT, R.T., CHOW, C.W., BLITZER, J.T., PITCHER, J.A., CLAING, A., STOFFE L, R.H., BARAK, L.S., SHENOLIKAR, S., WEINMAN, E.J., GRINSTEIN, S. & LEFKOWITZ, R.J. (1998). The β_2-adrenergic receptor interacts with the Na^+/H^+ exchanger regulatory factor to control Na^+/H^+ exchange. *Nature,* **392,** 626–630.

HANLEY, M.R. & IVERSEN, L.L. (1978). Muscarinic cholinergic receptors in rat corpus striatum and regulation of guanosine cyclic 3′,5′-monophosphate. *Mol. Pharmacol.,* **14,** 246–255.

HEBERT, T.E., MOFFETT, S., MORELLO, J.P., LOISE T.P. BICET, D.G., BARRETT, C. & BOUVIER, M. (1996). A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem.,* **271,** 16384–16392.

HILL, S.J., DAUM, P. & YOUNG, J.M. (1981). Affinities of histamine H_1-antagonists in guinea-pig brain: similarity of values determined from [^{3}H]mepyramine binding and from inhibition of a functional response. *J. Neurochem.,* **37,** 1357–1360.

HILL, S.J., YOUNG, J.M. & MARRIAN, D.H. (1977). Specific binding of ^{3}H-mepyramine to histamine H_1-receptors in intestinal smooth muscle. *Nature,* **270,** 361–363.

HOFFMAN, C., GAIETTA, G., BUNEMANN, M., ADAMS, S.R., OBERDOFF-MAAS, S., BEHR, B., VILARDAGA, J.P., TSIEN, R.Y., ELLISMAN, M.H. & LOHSE, M.J. (2005). A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat. Methods,* **2,** 171–176.

INSEL, P.A. & KENNEDY, M.S. (1978). Colchicine potentiates beta-adrenoreceptor-stimulated cyclic AMP in lymphoma cells by an action distal to the receptor. *Nature,* **273,** 471–473.

JORDAN, B.A. & DEVI, L.A. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature,* **399,** 697–700.

KAHOUT, T.A. & LEFKOWITZ, R.J. (2003). Regulation of G-protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol. Pharmacol.,* **63,** 9–18.

KENAKIN, T. (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J.,* **15,** 598–611.

KENAKIN, T. (2002). Drug efficacy at G protein-coupled receptors. *Ann. Rev. Pharmacol. Toxicol.,* **42,** 349–379.

KOENIG, J.A. & EDWARDSON, J.M. (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.,* **18,** 276–287.

LAZERENO, S., DOLEZAL, V., POPHAM, A & BIRDSALL, N.J.M. (2004). Thiochrome enhances acetylcholine affinity at muscarinic M4 receptors: receptor subtype selectivity *via* cooperativity rather than affinity. *Mol. Pharmacol.,* **65,** 257–266.

LEFKOWITZ, R.J. (2004). Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol. Sci.,* **25,** 413–422.

LEFKOWITZ, R.J., COTECCIA, S., SAMANA, P. & COSTA, T (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.,* **14,** 303–307.

LUTTRELL, L.M. & LEFKOWITZ, R.J. (2002). The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J. Cell Sci.,* **115,** 455–465.

LUTTRELL, L.M., ROUDABUSH, F.L., CHOY, E.W., MILLER, W.E., FIELD, M.E., PIERCE, K.L. & LEFKOWITZ, R.J. (2001). Activation and targetting of extracellular-signal-regulated kinases by β-arrestin scaffolds. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 2449–2454.

MAGGIO, R., VOGEL, Z. & WESS, J. (1993). Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular 'cross-talk' between G-protein-linked receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 3103–3107.

MARSHALL, F.H., JONES, K.A., KAUPMANN, K. & BETTLER, B (1999). $GABA_B$ receptors – the first 7TM heterodimers. *Trends Pharmacol. Sci.,* **20,** 396–399.

MCLATCHIE, L.M., FRASER, N.J., MAIN, M.J., WISE, A., BROWN, J., THOMPSON, N., SOLARI, R., LEE, M.G. & FOORD, S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-like receptor. *Nature,* **393,** 333–339.

MILANO, C.A., ALLEN, L.F., ROCKMAN, H.A., DOLBER, P.C., MCMINN, T.R., CHIEN, K.R., JOHNSON, T.D., BOND, R.A. & LEFKOWITZ, R.J. (1994). Enhanced myocardial function in transgenic mice overexpressing the beta 2-adrenergic receptor. *Science,* **264,** 582–588.

MORISSET, S., ROULEAU, A., LIGNEAU, X., GBAHOU, F., TARDIVEL-LACOMBE, J., STARK, H., SCHUNACK, W., GANELLIN, C.R., SCHWARTZ, J.C. & ARRANG, J.M. (2000). High constitutive activity of native H_3-receptors regulates histamine neurones in brain. *Nature,* **408,** 860–864.

NAHORSKI, S.R. (1977). Altered responsiveness of cerebral beta adrenoceptors assessed by adenosine cyclic 3′,5′-monophosphate formation and [^{3}H]propranolol binding. *Mol. Pharmacol.,* **13,** 679–689.

OSTROM, R.S. & INSEL, P.A. (2004). The evolving role of lipid rafts and caveolae in G protein-coupled receptor signalling: implications for molecular pharmacology. *Br. J. Pharmacol.,* **143,** 235–245.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. Roy. Soc. B.,* **163,** 1–44.

RAPACCIUOLO, A., SUVARNA, S., BARKI-HARRINGTON, L., LUTTRELL, L.M., CONG, M., LEFKOWITZ, R.J. & ROCKMAN, H.A. (2003). Protein kinase A and G-protein-coupled receptor kinase phosphorylation mediates β1-adrenergic receptor endocytosis through different pathways. *J. Biol. Chem.,* **278,** 35403–35411.

RODBELL, M. (1997). Signal transduction: evolution of an idea. In *Nobel Lectures, Physiology or Medicine 1991–1995,* Ringertz (ed). Singapore: World Scientific Publishing Co.

SCHULTZ, J. & DALY, J.W. (1973). Adenosine 3′,5′-monophosphate in guinea-pig cerebral cortical slices: effects of alpha- and beta-adrenergic agents, histamine, serotonin and adenosine. *J. Neurochem.,* **21,** 573–579.

SEAMON, K.B. & DALY, J.W. (1981). Guanosine-5′-(β,γ-inido)triphosphate inhibition of forskolin-activated adenylate cyclase is mediated by the putative inhibitory gunine nucleotide regulatory protein. *J. Biol. Chem.,* **257,** 11591–11596.

SEAMON, K.B., PADGETT, W. & DALY, J.W. (1981). Forskolin: unique diterpine activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. U.S.A.,* **78,** 3363–3367.

SELBIE, L.A. & HILL, S.J. (1998). G-protein-coupled receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol. Sci.,* **19,** 87–93.

SELF, T.J., OAKLEY, S.M. & HILL, S.J. (2005). Clathrin-independent internalization of the human histamine H_1-receptor in CHO-K1 cells. *Br. J. Pharmacol.,* **146,** 612–624.

SETA, K., NANAMORI, M., MODRALL, J.G., NEUBIG, R.R. & SADOSHIMA, J. (2002). AT1 receptor mutant lacking heterotrimeric G protein coupling activates the Src-Ras-ERK pathway without nuclear translocation of ERKs. *J. Biol. Chem.,* **277,** 9268–9277.

SHENOY, S.K., MCDONALD, P.H., KAHOUT, T.A. & LEFKOWITZ, R.J. (2001). Regulation of receptor fate by ubiquitination of activated beta-2 adrenergic receptor and beta-arrestin. *Science,* **294,** 1307–1313.

SMIT, M.J., LEURS, R., ALEWIJNSE, A.E., BLAUW, J., VAN NIEUW AMEWRONGEN, G.P., VAN DE VREDE, Y., ROOVERS, E. & TIMMERMAN, H. (1996). Inverse agonism of histamine H_2 antagonists accounts for upregulation of spontaneously active histamine H_2 receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 6802–6807.

SOUDIJN, W., VAN WIJNGAARDEN, I. & IJZERMAN, A.P. (2004). Allosteric modulation of G protein-coupled receptors: perspectives and recent developments. *Drug Discovery Today,* **9,** 752–758.

STEPHENSON, R.P. (1956). Modification of receptor theory. *Br. J. Pharmacol. Chemother.,* **11,** 379–393.

STEVENS, P.A., PEDIANA, J., CARILLO, J.J. & MILLIGAN, G. (2001). Coordinated agonist regulation of receptor and G protein palmitoylation and functional rescue of palmitoylation-deficient mutants of the G protein G_{11Q} following fusion to the α_{1B}-adrenoceptor. *J. Biol. Chem.,* **276,** 35883–35890.

STRANGE, P.G., BIRDSALL, N.J.M. & BURGEN, A.S.V. (1978). Ligand-binding properties of the muscarinic acetylcholine receptor in mouse neuroblastoma cells. *Biochem. J.,* **172,** 495–501.

SUNDARAM, H., NEWMAN-TANCREDI, A. & STRANGE, P.G. (1993). Characterization of recombinant human serotonin 5HT1A receptors expressed in Chinese hamster ovary cells. [^{3}H]spiperone discriminates between the G-protein-coupled and -uncoupled forms. *Biochem. Pharmacol.,* **45,** 1003–1009.

TAN, C.M., BRADY, A.E., NICKOLS, H.H., WANG, Q. & LIMBIRD, L.E. (2004). Membrane trafficking of G protein-coupled receptors. *Ann. Rev. Pharmacol. Toxicol.,* **44,** 559–609.

TOBIN, A.B., TOTTY, N.F., STERLIN, A.E. & NAHORSKI, S.R. (1997). Stimulus-dependent phosphorylation of G protein-coupled receptors by casein kinase 1α. *J. Biol. Chem.,* **272,** 20844–20849.

WEI, H., AHN, S., SHENOY, S.K., KARNIK, S.S., HUNYADI, L., LUTTRELL, L.M. & LEFKOWITZ, R.J. (2003). Independent β-arrestin2 and G-protein-mediated pathways for angiotensin II activation of extracellular signal regulated kinases 1 and 2. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 10782–10787.

WISE, A, JUPE, S.C. & REES, S (2004). The identification of ligands at orphan G-protein coupled receptors. *Ann. Rev. Pharmacol. Toxicol.,* **44,** 43–66.

WREGGETT, K.A. & DE LEAN, A. (1984). The ternary complex model. Its properties and application to ligand interactions with the D2-dopamine receptor of the anterior pituitary gland. *Mol. Pharmacol.,* **26,** 214–227.