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REVIEW

Exploring the dynamics of regulation of G protein-coupled receptors using green fluorescent protein

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Abbreviations: BRET, bioluminescence resonance energy transfer; CEC, chlorethylclonidine; [^{3}H]CGP-12177, (−)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidizol-2-one; [^{3}H]DHA, [^{3}H]dihydroalprenolol; FACS, fluorescence activated cell sorting; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GnRH, gonadotrophin releasing hormone; GRK, G protein-coupled receptor kinase; NHERF, Na^+/H^+ exchanger regulatory factor; TRH, thyrotropin releasing hormone; TRHR-1, thyrotropin releasing hormone receptor-1

Introduction

New advances in understanding the details of cellular response to agonist ligands require integration of approaches derived from pharmacology and both cell and molecular biology. Direct visualization of alterations in the concentration and distribution gradients of [Ca^{2+}] in individual cells achieved by monitoring differences in fluorescence characteristics of Ca^{2+} indicator dyes in their Ca^{2+}-bound and unbound states has produced an almost unimaginable increase in our understanding of the dynamics of cellular Ca^{2+} handling over the last ten years. However, until relatively recently the potential for non-invasive analysis of the dynamics of trafficking of cellular proteins seemed limited. In the recent past the use of mutationally modified forms of the autofluorescent green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has begun to make imaging of many proteins in living single cells a reality. In the near future this is likely to result in as profound a revolution in our understanding of cellular targeting, trafficking and interactions between proteins of pharmacological interest as was the introduction and improvement of Ca^{2+} indicator dyes for the regulation of Ca^{2+} homeostasis. Although this approach has now been used for a wide range of signalling proteins this review will concentrate on insights gained from the use of GFP-tagging into the kinetics and dynamics of regulation of G protein-coupled receptors (GPCRs) and proteins which interact directly with them.

Until the introduction of GFP as an experimental tool single, intact, cell detection and analysis of the potential cellular redistribution of a GPCR or other GPCR-interacting proteins was restricted to two approaches. In the first of these pharmacophores from ligands with appropriate pharmacological selectivity were modified to incorporate fluorescent chemical groups. Key issues for this approach are that pharmacological selectivity and, if possible, potency are preserved in the modified ligand. Validation of the ligands for potential use in physiologically relevant systems could be achieved initially using cell lines which had been transfected with cDNAs for appropriate GPCRs (Daly *et al*., 1998). However, unless a distinct increase in fluorescence of the ligand is obtained upon binding the GPCR in question, direct visualization is difficult to achieve without substantial washing of the cell or tissue. For GPCRs with small molecule endogenous ligands the ensuing limitation on the affinity of drugs which can be used generally results in the most appropriate ligands being antagonists. As such, a rather static picture of GPCR expression and distribution is the most likely outcome. Many of these issues have recently been reviewed and assessed (McGrath *et al*., 1996). In the case of GPCRs with high affinity peptide ligands it is often possible to attach a suitable fluorescent dye to a location in the peptide not involved in an integral way in binding the receptor. Ligand affinity is thus often preserved. Furthermore, given the fact that agonist binding to a GPCR frequently results in an alteration in the cellular distribution of the GPCR and the fact that high affinity peptide ligands are routinely co-internalized with the GPCR owing to their slow dissociation rates, internalization of the peptide-GPCR complex can be monitored directly (see Ashworth *et al*., 1995 for example). Although many early studies utilized fluorescent tags such as rhodamine, improvements in chemistry in this area have allowed generation of fluorescently-tagged peptides which are much brighter and which can thus be used to localize and visualize GPCRs expressed at relatively low levels (see Advanced Bioconcepts at http://www.bioconcept.com for example).

The second approach utilizes immunocytochemical detection of the GPCRs. Appropriate antisera, generally to peptide sequences of the GPCR in question, have been produced in many cases and represent the only practical means to immunolocalize GPCRs in tissues and cells which express the GPCR endogenously. However, by far the most widespread strategy in cell transfection studies has been the use of epitope-tagging. Herein, a cDNA encoding a GPCR is modified to incorporate a sequence, either within the N-terminal extracellular region or at the C-terminus, which is known to be identified by a well defined, high affinity, antibody. Widely used epitope tags include HA, c-*myc*, FLAG™ and VSV. With such modified GPCRs it is important to define that the alterations to the protein sequence do not modify the pharmacology of the GPCR. For GPCRs with small molecule natural ligands, N-terminal epitope tagging is unlikely to modify ligand binding or function. C-terminal tagging would also be anticipated to have little effect on ligand pharmacology

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or on second messenger regulation by a GPCR. However, recent studies have suggested that not all functions of a GPCR necessarily arise *via* G protein activation. For example Hall *et al*. (1998) have recently demonstrated that the β_2-adrenoceptor interacts with the Na^+/H^+ exchanger regulatory factor (NHERF) *via* a PDZ domain in NHERF. Mutagenesis studies indicate that it is the extreme C-terminal motif of the β_2-adrenoceptor which is responsible for this interaction. Although it has not been examined directly, it must be anticipated that addition of a C-terminal epitope tag to the β_2-adrenoceptor would modify or potentially ablate this interaction. Immunofluorescence pictures of such epitope-tagged GPCRs before and following treatment with different agonist ligands have provided direct and key insights into the regulation of GPCR internalization and trafficking. However, by their very nature, such experiments must be performed on fixed and permeabilized cells (to allow antibody access to the potentially internalized GPCR) and thus limits use in real time, dynamic, cell imaging.

Green fluorescent protein

The *Aequorea victoria* photoprotein GFP is a 238 amino acid protein that emits green light with an emission maximum of 509 nm upon fluorescent excitation at 488 nm. Unlike other bioluminescent reporter proteins no additional substrates or cofactors are required for light emission (Chen *et al*., 1995). GFP fluorescence is stable and has been measured non-invasively in living cells of many species including mammals, drosophila, *C. elegans*, yeast and *E. coli*. GFP fluorescence can be detected by fluorimetry, by fluorescence activated cell sorting (FACS) and by microscopy. As there is no required cofactor the attractiveness of GFP as a reporter protein is obvious (Chalfie & Kain, 1998). The availability of the cDNA sequence for GFP has resulted in the generation and characterization of several GFP mutants with enhanced fluorescence emission. Mutation of the serine at amino acid 65 to threonine resulted in the generation of a protein with a 6 fold increase in the intensity of fluorescence emission (Haas *et al*., 1996). Furthermore, the presence of the $Ser^{65}Thr$ and mutation of the phenylalanine residue at position 64 to leucine has resulted in a 35 fold increase in fluorescence intensity (Haas *et al*., 1996). In addition, a number of novel mutants of GFP have also been identified with altered excitation or emission characteristics. For example, mutation of the tyrosine residue at position 66 to histidine generated a protein with altered spectral properties and blue fluorescence emission. This so-called blue fluorescent protein has a λ_{max} for excitation of 458 nm and for emission of 480 nm (Chalfie & Kain, 1998). These and many other variants of GFP are now commercially available from a number of sources.

Studies using GPCR–GFP fusion constructs

Although often used directly in cellular transient transfection studies simply to identify cells in a field which have taken up cDNA and express the protein, the true benefit of GFP can be utilized following its construction into fusion proteins to tag and thus monitor the distribution of another protein of interest. The modified forms of GFP described above have been widely used in fusion proteins to assess protein trafficking (Ferrer *et al*., 1997), and subcellular localization of recombinantly expressed proteins (Wang and Hazelrigg, 1994). Introduction of this technology into analysis of GPCR regulation was first reported at the end of 1997 (Barak *et al*., 1997). Since then a number of groups have described the creation and use of GPCR–GFP fusion proteins to monitor both receptor internalization and recycling following agonist treatment and to explore a number of distinct pharmacological issues.

As GFP is a 27 kDa protein it is greater than 50% of the size of the majority of family 1 and family 2 GPCRs. As such, a key initial issue must be to ensure that the basic features of GPCRs are not altered by their tagging with GFP. All the constructs used to date have the GFP linked to the C-terminal tail of the GPCR. When expressed alone, GFP is a soluble protein which folds as a tightly packed globular structure. It is thus unlikely to allow appropriate targeted expression of the fusion protein were GFP to be linked to the N-terminus of a GPCR (unless an appropriate leader sequence was also incorporated into the cDNA) as this would require transit of the GFP across the plasma membrane. Encouragingly, in all reports to date the GFP-tagged GPCRs have been able to regulate appropriate second messenger production in an agonist-dependent manner. Furthermore, clear evidence for agonist-mediated phosphorylation of GFP-tagged GPCRs and their interaction with proteins of the arrestin family has been produced.

As with many areas of GPCR research, one of the initial reports on the regulation of a GFP-tagged GPCR utilized the human β_2-adrenoceptor (Barak *et al*., 1997). Following transient expression in HEK293 cells a fusion protein between the β_2-adrenoceptor and a mutated form of GFP with enhanced autofluorescence displayed similar agonist and antagonist binding characteristics as the wild type β_2-adrenoceptor. Furthermore, the fusion protein also allowed concentration-dependent stimulation of cyclic AMP production which was desensitized following short-term exposure to a high concentration of isoprenaline. Thus, although no very extensive pharmacological comparison of an isolated GPCR and an equivalent GFP-tagged form of the same GPCR has yet been published, basic features of GPCR function appear well maintained. Both the isolated β_2-adrenoceptor and the GPCR–GFP fusion protein could be phosphorylated in an agonist-dependent manner. Although it was not formally demonstrated that the sites of phosphorylation in the fusion protein were the same as those modified in the isolated GPCR, or indeed whether the GFP tag also became phosphorylated, both forms of the GPCR became internalized to similar extents following a 30 min challenge with isoprenaline. The β_2-adrenoceptor-GFP fusion protein became internalized into defined vesicles in response to agonist, a process which could be followed in intact cells and in real time. The capacity to photobleach regions of a cell membrane on exposure to a short pulse of high intensity light has been widely applied to examine the diffusional mobility of proteins in membranes. Such fluorescence recovery after photobleaching (FRAP) monitoring of the movement of the β_2-adrenoceptor-GFP construct from outwith the photobleached area into this region allowed calculation of the rate of diffusion of the GPCR as $4-12 \times 10^{-9}$ cm^2 sec^{-1}.

A significant issue with transient transfection of many GPCRs into commonly used cell lines is that when expression of the protein is driven by a strong viral promoter, a significant proportion of the protein so produced appears to remain trapped in the Golgi apparatus or other intracellular membranes and is not effectively trafficked to the plasma membrane. This can be visualized directly when using a GPCR–GFP fusion protein (see Milligan, 1998, for example). Such inappropriately trafficked protein poses obvious difficulties for detailed analysis of agonist regulation of the subcellular distribution of GFP-tagged GPCRs in such transiently

transfected cells and thus the production of cell clones stably expressing the fusion protein is to be preferred. In the case of the β_2-adrenoceptor this has been achieved in both HeLa (Kallal *et al*., 1998) and HEK293 (McLean & Milligan, 1999) cells (Figure 1). In HeLa cells expressing some 200 fmol mg^{-1} membrane protein of β_2-adrenoceptor-GFP, Kallal *et al.* (1998) recorded a rapid sequestration of this construct in response to isoprenaline which could be monitored in parallel by a reduction in levels of intact cell specific binding of the hydrophillic antagonist [^{3}H]CGP – 12177. HEK293 cell derived clones stably expressing higher levels of a β_2-adrenoceptor-GFP (5.5 pmol mg^{-1} membrane protein) were examined by McLean & Milligan (1999) but similar agonist-induced sequestration was observed in both [^{3}H]CGP – 12177 binding studies and by visual examination in a confocal microscope. In both systems internalization of β_2-adrenoceptor-GFP induced by short-term exposure to agonist could be reversed by removal of agonist and its replacement with the antagonist alprenolol. In the HeLa cell system long-term treatment (up to 24 h) with a maximally effective concentration of isoprenaline resulted in downregulation of up to 50% of the receptor binding sites as monitored by the binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA) (Kallal *et al*., 1998). By contrast, equivalent treatment of the HEK293 clone could be shown to largely remove plasma membrane localized β_2-adrenoceptor-GFP, but direct visualization of the cells indicated that much of the internalized β_2-adrenoceptor-GFP was not degraded effectively. This pictorial representation was effectively supported by intact cell [^{3}H]DHA binding assays (this ligand is hydrophobic and thus can monitor total cellular levels of the fusion protein in intact cells) (McLean & Milligan, 1999). This may reflect an overload of the degradative pathway resulting from the high levels of expression of the β_2-adrenoceptor-GFP in these cells, a stabilization effect of attachment of GFP to the GPCR or some combination thereof.

Although only qualitative data could be provided, it is of considerable interest to note that the rate and extent of internalization of β_2-adrenoceptor-GFP in HeLa cells may be determined by the duration of action and intrinsic activity of the agonist ligand used. Mathematical predictions of the extent and rate of GPCR internalization have been considered (Koenig & Edwardson, 1997). However, the capacity to directly visualize these processes adds substantially to understanding. Kallal *et al.* (1998) noted much slower internalization of the fluorescent protein in response to the low intrinsic activity, but long acting, β_2-adrenoceptor agonist salmeterol than in response to isoprenaline. Preliminary studies have also compared internalization of a human β_1-adrenoceptor-GFP construct stably expressed at some 5 pmol mg^{-1} membrane protein in HEK293 cells with the β_2-adrenoceptor-GFP in the same cell system discussed above. Isoprenaline-induced internalization was both slower and less dramatic for the human β_1-adrenoceptor-GFP fusion protein (McLean & Milligan, 1999). The internalized construct could also be recycled to the plasma membrane, however, following removal of isoprenaline and its replacement by the β_1-adrenoceptor-selective blocker betaxolol (McLean & Milligan, 1999).

A considerable series of other GPCR – GFP fusion proteins have been constructed and various aspects of cellular distribution and agonist-dependent regulation examined following either transient or stable expression in mammalian cell lines. These include the cholecystokinin type A(CCK_1) receptor (Tarasova *et al*., 1997), the thyrotropin releasing-hormone receptor-1 (TRHR-1) (Milligan, 1998; Drmota *et al*., 1998a, 1999), the α_{1A} (Hirasawa *et al*., 1997) and α_{1B}-adrenoceptors (Hirasawa *et al*., 1997; Awaji *et al*., 1998), the vasopressin V2 receptor (Schulein *et al*., 1998), the parathyroid hormone receptor (Conway *et al*., 1999), the CXCR-1 chemokine receptor (Barlic *et al*., 1999) and the Ca^{2+}-sensing receptor (Gama & Breitwieser, 1998).

Many of these studies have explored the likely nature of intracellular vesicles into which the agonist-activated receptor is translocated by taking advantage of the opportunity to 'co-localize' as a 'yellow' image the internalized GPCR – GFP fusion protein with makers known to enter the cells *via* the constitutive endocytic pathway (Clague, 1998). By providing cells with rhodamine, Cy3, Texas red, Alexa^{594} or other appropriately labelled forms of transferrin, followed by washing to remove excess label, overlap between the transferrin-containing vesicles and those containing the internalized GPCR – GFP fusion protein is obtained by merging of the 'red' and 'green' images (Tarasova *et al*., 1997; Drmota *et al*., 1998a; Kallal *et al*., 1998; Awaji *et al*.,

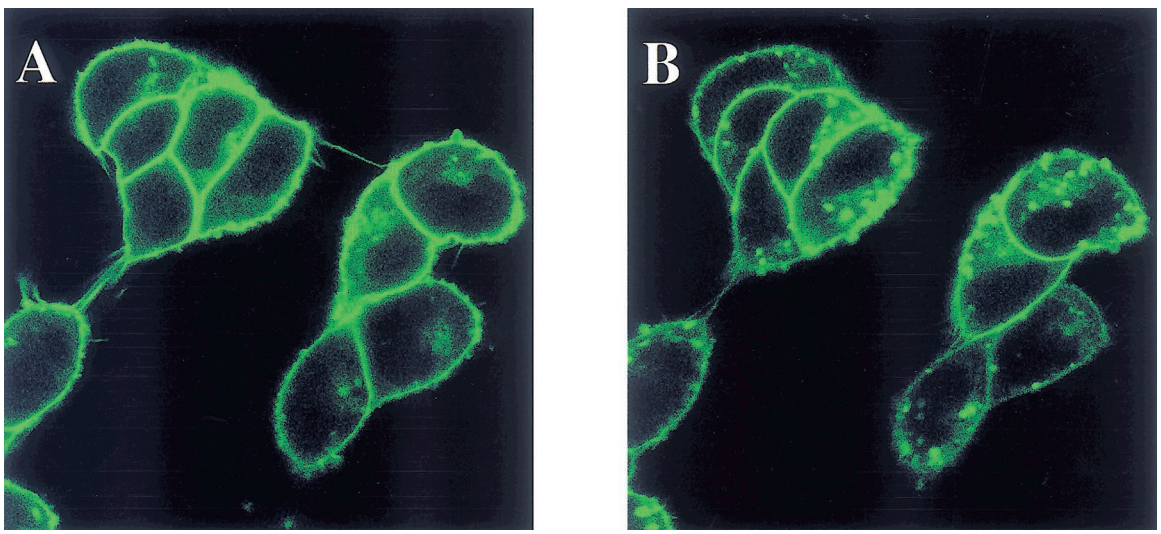

Figure 1 Internalisation of a β_2-adrenoceptor-GFP fusion protein in response to isoprenaline. The human β_2-adrenoceptor was C-terminally tagged with GFP and expressed stably in HEK293 cells. A field of cells expressing this construct was visualised with a confocal microscope before (A) and following application of isoprenaline (10 μM, 30 min) (B) (see McLean & Milligan, 1999 for further details).

1998). Similar strategies with appropriately labelled forms of Dextran or other molecules which are preferentially sorted to late endosomes and lysosomes can then be used to study the delivery of GPCR–GFP fusion proteins to these compartments with sustained agonist treatment of cells (Kallal *et al*., 1998). It is interesting to note that stable expression of a CCK_1 receptor-GFP construct in three different, widely used, cell lines resulted in predominantly agonist-dependent fusion protein internalization in CHO and HeLa cells. However, this process was largely constitutive in NIH3T3 cells (Tarasova *et al*., 1997). As this constitutive process was blocked by CCK_1 receptor antagonists then the selection of cell system may be predicated by whether the investigator wishes to examine the function of agonist or antagonist ligands.

Although impractical to display in a short series of single fixed snapshots, a number of journals now accept supplementary, multimedia information, which is posted on a web site in conjunction with a published paper. This provides an opportunity to generate short video movies of agonist-induced alteration in the cellular distribution of a GPCR–GFP fusion protein (Drmota *et al*., 1999, see http://www.BiochemJ.org/bj/340/bj3400529add.htm for further details). Such videos allow the best representation of the dynamics of protein movement and a number of groups also maintain such videos on personal web sites. In the case of a TRHR-1–GFP fusion protein expressed stably in HEK293 cells, initially this has homogenous distribution around the plasma membrane (Figure 2). Addition of TRH results in rapid concentration of the GFP signal to punctate foci at the plasma membrane. This is followed by internalization of the construct into a large number of small punctate vesicles closely apposed to the plasma membrane (Figure 2). At later time points many of these small vesicles appear to fuse into larger structures (Drmota *et al*., 1999). The internalization of TRHR-1–GFP, but not its recruitment to foci at the plasma membrane, is blocked by hypertonic sucrose (Drmota *et al*., 1998a). As this treatment is routinely used to block internalization of proteins *via* clathrin-coated pits it is tempting to assume that the plasma membrane foci represent such regions.

Using combinations of imaging of a GPCR–GFP and immunolocalization in fixed cells of G protein α subunits it has also been possible to address the possibility of co-internalization of a GPCR and an associated G protein. Using HEK293 cells expressing high levels of both the rat TRHR-1 and the G protein $G_{11}\alpha$, Drmota *et al*. (1998b) were able to use immunofluorescence approaches to observe that sustained treatment with a maximally effective concentration of TRH resulted in movement of this G protein away from the plasma membrane and into punctate intracellular structures. However, by following the internalization of the TRHR-1 in intact cell [^{3}H]TRH binding studies the movement of the G protein was substantially slower than of the GPCR (Drmota *et al*., 1998b). To extend this work and assess potential co-localization of the GPCR and G protein, Drmota *et al*. (1999) concurrently examined the cellular distribution of TRHR-1–GFP and the phospholipase C-linked G proteins $G_q\alpha$ and $G_{11}\alpha$ using a confocal microscope. Prior to TRH treatment both sets of proteins were heavily concentrated at the plasma membrane and merging of the images confirmed their overlap. Following sustained treatment with TRH (1 μM, 4 h) the bulk of TRHR-1–GFP had been internalized. Although a large fraction of the cellular $G_q\alpha$ and $G_{11}\alpha$ remained at the plasma membrane, clear evidence was also produced for internalization of these G proteins. Merging of the images of TRHR-1–GFP and $G_q\alpha$ and $G_{11}\alpha$ location indicated the internalized proteins to be in overlapping, intracellular membrane fractions. When the kinetics of this process were examined internalization of TRHR-1–GFP was rapid (5–10 min) but it was not possible to measure G protein internalization within 1 h. At this juncture it is impossible to know whether this observed temporal dislocation indicates that the different proteins are really internalized with very different kinetic (and thus potentially mechanistic) bases or whether the observations reflect the high levels of G protein in these cells compared to TRHR-1–GFP. It is possible that internalization of TRHR-1–GFP is always associated with co-internalization of $G_q\alpha$ and $G_{11}\alpha$ but that many cycles of GPCR internalization and recycling to the plasma membrane are required to internalize a significant fraction of the G protein (which may not recycle) such that internal co-localization can be imaged. Future studies which interfere with GPCR–GFP recycling may address this issue.

As well as phosphorylation in a strictly agonist-dependent manner, which is produced by members of the G protein-coupled receptor kinase (GRK) family, many GPCRs can be phosphorylated *via* second messenger-activated kinases to provide a potential for heterologous desensitization. As the α_{1b}-adrenoceptor becomes phosphorylated in response to treatment with phorbol esters (Diviani *et al*., 1997) this is presumably mediated *via* isoforms of protein kinase C. Following expression in the gonadotroph-derived cell line αT3-1 the effects of various pharmacological agents on the internalization of an α_{1b}-adrenoceptor-GFP construct was investigated (Awaji *et al*., 1998). The phospholipase C inhibitor U73,122 (10 μM, 10 min pretreatment) was largely able to prevent noradrenaline-promoted internalization of the construct as was the rather broad spectrum protein kinase inhibitor staurosporine, which is often inappropriately used as a selective protein kinase C inhibitor. Although not as effective as noradrenaline, the protein kinase C activator phorbol myristate acetate (1 μM, 30 min treatment) was also able to stimulate internalization of α_{1b}-adrenoceptor-GFP. Furthermore, heterologous internalization of the α_{1b}-adrenoceptor-GFP construct was also observed by treatment of the cells with gonadotrophin releasing hormone (GnRH) to activate the endogenously expressed, PLC-coupled, GnRH receptor (Awaji *et al*., 1998).

Not all GPCRs are effectively delivered to the plasma membrane of cells. Hirasawa *et al*. (1997) have shown that although transient expression of an α_{1B}-adrenoceptor-GFP construct in COS-7 cells results in the bulk of the expressed protein reaching the plasma membrane, this was not true for an equivalent GFP-tagged form of the α_{1A}-adrenoceptor. They used this phenomenon to re-examine the long held suggestion that the hydrophillic α_1-adrenoceptor alkylating agent chlorethylclonidine (CEC) can be used as a selective pharmacological discriminator for the α_{1B}-adrenoceptor. Inactivation of the two GPCR subtypes by CEC was equivalent in membrane preparations but displayed selective inactivation of the α_{1B}-adrenoceptor in intact cell studies. They thus concluded that CEC is not inherently a selective alkylating agent between these two closely related GPCRs if provided with equal access, but that observed differential effects of CEC might rather relate to uneven cellular distribution of the two GPCRs (Hirasawa *et al*., 1997). GPCR mutagenesis can often result in poor plasma membrane delivery of the modified protein. In experiments designed to explore and contrast features of the agonist binding pocket and the capacity of a GPCR to adopt a conformation capable of G protein activation it is important to be able to ascertain whether the modified GPCR has been expressed and properly targeted. Although yet to be

employed in exactly this context, GFP-tagging of mutationally modified forms of the vasopressin V2 receptor has been used to explore the contribution of a dileucine sequence, and an associated upstream acidic residue often found within such dileucine motifs, in the C-terminal tail of this GPCR to the capacity of the expressed protein to escape from the endoplasmic reticulum (Schulein *et al*., 1998). Another potentially useful situation in which GFP-tagging of GPCRs has yet to be utilized in a significant manner is to confirm the expression and appropriate targeting of 'orphan' GPCRs for which ligands are currently unknown, when they are being incorporated into ligand discovery programmes.

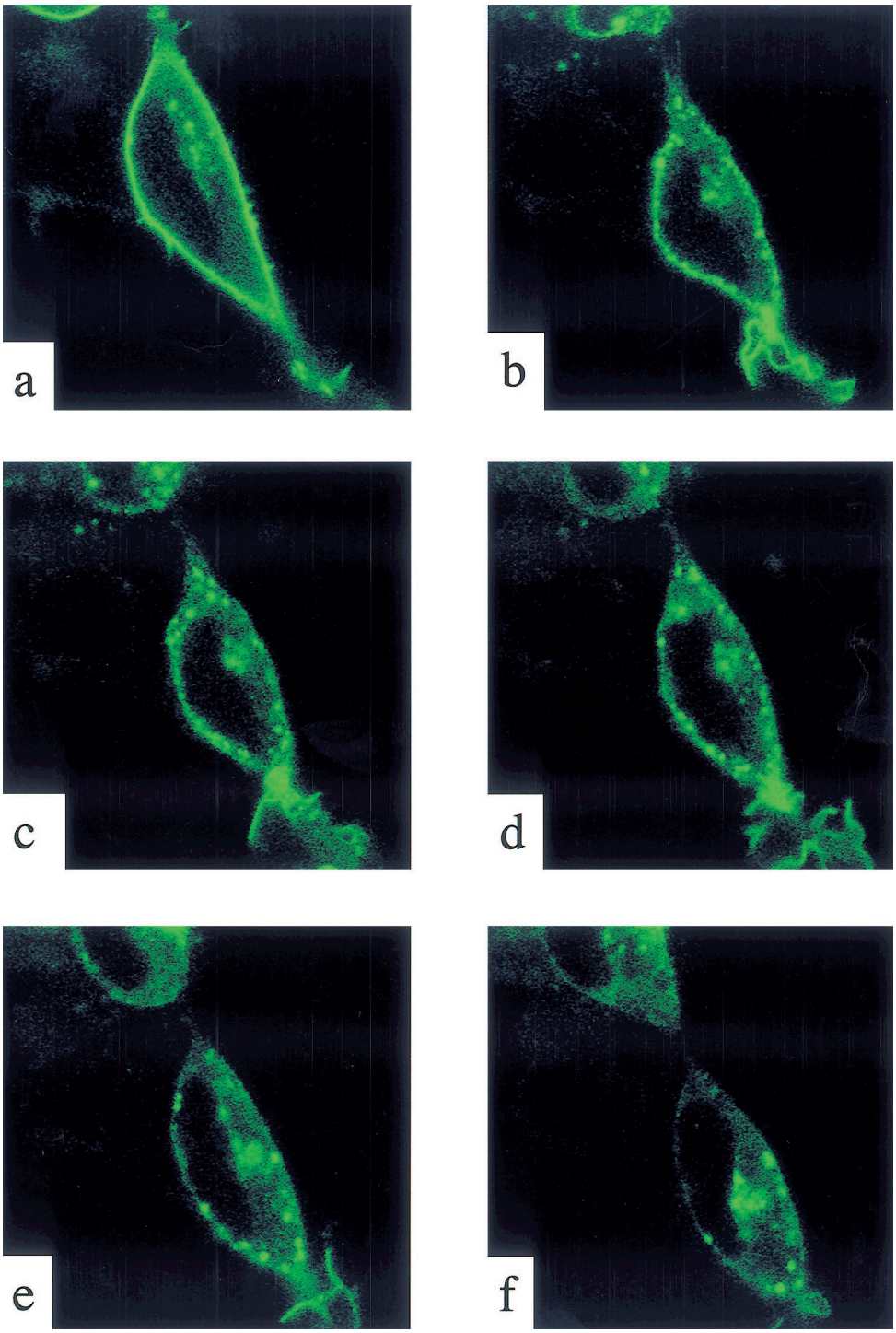

Figure 2 Agonist-induced cellular redistribution of a GFP-tagged form of the long isoform of the rat thyrotropin-releasing hormone receptor-1. An N-terminally VSV epitope-tagged form of the long isoform of the rat thyrotropin-releasing hormone (TRH) receptor-1 was C-terminally tagged with GFP and expressed stably in HEK293 cells. Cells of clone VTGP1 (Drmota *et al*., 1998) were grown on a glass cover slip and imaged in a confocal microscope at time 0 (a) and after addition of 10 μM TRH for 4 (b), 7.5 (c), 8.5 (d), 12 (e) and 23 (f) min. The construct moved from an essentially homogenous plasma membrane distribution to an initially plasma membrane-associated punctate distribution to location in intracellular punctate vesicles some of which became perinuclear associated (see text for details). Pictures are taken from Drmota *et al*. (1999) with permission.

Studies using G protein–GFP fusion constructs

The most obvious set of GPCR interacting proteins are the heterotrimeric G proteins. Rather little has been done, however, with GFP-tagged forms of G proteins. This may reflect that the extreme C-terminus of G protein α subunits represents a key contact site for interaction with GPCRs (Bourne, 1997; Milligan & Rees, 1999). As such, it has yet to be demonstrated that a G protein α subunit-GFP fusion protein, in which the GFP is attached to the C-terminal tail of the G protein, would be able to interact effectively with a GPCR. Equally, all G protein α subunits are either co- or post-translationally modified in their N-terminal region by attachment of saturated fatty acyl chains. For the G_i-family G proteins myristate is added to the N-terminal glycine (Gly^2) exposed after removal of the initiator methionine. This cannot occur if this glycine is no longer at the N-terminus, as would be the case if GFP was fused in front of the G protein. Palmitate and other saturated fatty acids are added to Cys^3. As such, the only significant use to date of GFP-tagged forms of a G protein α subunit has been to explore the role of N-terminal acylation in targeting the G protein both to the plasma membrane and more specifically to particular membrane subdomains. Galbiati *et al.* (1999) used C-terminally attached GFP as a reporter for these processes following construction of fusion proteins between either full-length $G_{i1}\alpha$ or a series of C-terminally truncated forms of this G protein. These were either wild-type at their N-terminus or mutationally modified (Gly^2Ala, Cys^3Ser) to prevent myristoylation and/or palmitoylation. Even constructs which contained only the N-terminal 32 amino acids of $G_{i1}\alpha$ were able to be appropriately modified by palmitate and myristate, and when both fatty acids were present the construct was appropriately targeted to the plasma membrane upon transient expression in HEK293 cells. However, mutational prevention of acylation either compromised (palmitoylation negative, Cys^3Ser) or abolished (combined myristate and palmitate negative, Gly^2Ala) plasma membrane targeting. Biochemical analysis of subcellular localization of these GFP containing constructs following expression in COS-7 cells indicated co-fractionation of a significant amount of the fully acylated G protein-GFP constructs with fractions heavily enriched for caveolin-1, a key marker protein for morphologically defined caveolae. By contrast, only a small fraction of the myristoylated but palmitoylation-resistant construct was present in the same fractions and the fully acylation-resistant constructs failed to co-fractionate with caveolin-1. Fully acylated $G_{i1}\alpha$-GFP constructs could be co-immunoprecipitated with wild-type caveolin-1 and this interaction also required the palmitoylation of caveolin-1 as no co-immunoprecipitation of acylated $G_{i1}\alpha$-GFP could be achieved following co-expression of the G protein construct and a palmitoylation resistant mutant of caveolin-1 (Galbiati *et al.*, 1999). Such results demonstrate a key role for N-terminal G protein acylation in appropriate cellular targeting of a G protein α subunit and for interaction with caveolin-1.

There has been considerable interest in reports that the subcellular localization of $G_{i2}\alpha$ and $G_{i3}\alpha$ is very distinct. $G_{i2}\alpha$ is targeted to the plasma membrane in human colon cancer HT-29 cells while $G_{i3}\alpha$ is targeted to the Golgi and endoplasmic reticulum (Petiot *et al.*, 1999). Although such studies to date have been performed by immunofluorescence, GFP fusion proteins offer an alternative approach. Furthermore, commercially available anti-GFP antibodies are effective in immunoprecipitation protocols. As such, these constructs would offer a means to explore potential differences in proteins which may selectively interact with these two G proteins to help define the basis for the observed differential cellular localizations.

Studies using β-arrestin-GFP fusion constructs

As noted previously, many GPCRs are phosphorylated, in a strictly agonist-dependent manner, at a number of Ser and Thr residues in their C-terminal tail by members of the GRK family of protein kinases. This however, represents only the initial phase of preventing GPCR and G protein interactions which results in desensitization of agonist function. The phosphorylated GPCR then acts as a binding partner for proteins of the arrestin family (Zhang et al, 1998a). In the unstimulated cell arrestins are cytoplasmic proteins. As such, it should be possible to image the movement of a GFP-tagged arrestin from the cytoplasm to the location of a GPCR at the plasma membrane in response to agonist. Recently, an elegant demonstration of this was achieved for an N-terminally HA epitope-tagged form of the human β_2-adrenoceptor and β-arrestin 1 in HEK293 cells. These studies also provided direct evidence for a role of c-Src in this process (Luttrell *et al.*, 1999). Efforts to demonstrate such interactions using GFP-tagged forms of β-arrestins were initiated by Barak *et al.* (1997) who generated a fusion protein in which a form of GFP with enhanced autofluorescence was attached to the C-terminus of β-arrestin 2. Transient co-transfection of the human β_2-adrenoceptor and β-arrestin 2-GFP into HEK293 cells followed by treatment of the cells with isoprenaline (20 μM, 10 min) resulted in translocation of a fraction of β-arrestin 2-GFP from a uniformly distributed cytoplasmic fluorescence to produce a halo of plasma membrane concentrated β-arrestin 2-GFP. Both the extent and kinetics of agonist-induced translocation were enhanced by co-transfection of GRK2. Equivalent results were produced with transient expression of the dopamine D_{1A} receptor (Barak *et al.*, 1997) and in review (Ferguson, 1998; Ferguson *et al.*, 1998) these authors have indicated that the basic features of agonist-induced β-arrestin 2-GFP translocation can be observed for a wide range of GPCRs. One GPCR which is highly resistant to agonist-induced short-term desensitization is the gonadotrophin releasing hormone (GnRH) receptor. This GPCR is unique in that, at least in mammalian species, it lacks a C-terminal tail. As such, it was anticipated not to be regulated by GRKs and β-arrestins. Following transient introduction of a β-arrestin 1-GFP fusion protein into HEK293 cells stably expressing high levels of the rat GnRH receptor, addition of GnRH failed to cause translocation of β-arrestin 1-GFP to the plasma membrane (Vrecl *et al.*, 1998). In contrast, equivalent transfection of HEK293 cells stably expressing the rat TRHR-1 resulted in rapid and effective translocation of β-arrestin 1-GFP to the plasma membrane upon addition of TRH (Milligan *et al.*, 1998; Vrecl *et al.*, 1998; Groarke *et al.*, 1999). It will be of interest to examine whether addition of the C-terminal tail of the TRHR-1 to the GnRH receptor will allow agonist-mediated rapid desensitization and interaction with β-arrestin 1-GFP. To extend such analyses Groarke *et al.* (1999) constructed HEK293 cell lines stably expressing β-arrestin 1-GFP and then transiently introduced a N-terminally VSV epitope-tagged form of the TRHR-1 (Figure 3). As anticipated, in the absence of agonist β-arrestin 1-GFP displayed a uniform cytoplasmic distribution. Combined confocal imaging of the β-arrestin 1-GFP and immunofluorescence detection of the GPCR indicated them to be in entirely resolved cellular locations. Addition of TRH resulted in a rapid (<1 min) movement of β-arrestin 1-GFP to the plasma membrane where merging of the signals of GPCR and β-arrestin 1-GFP

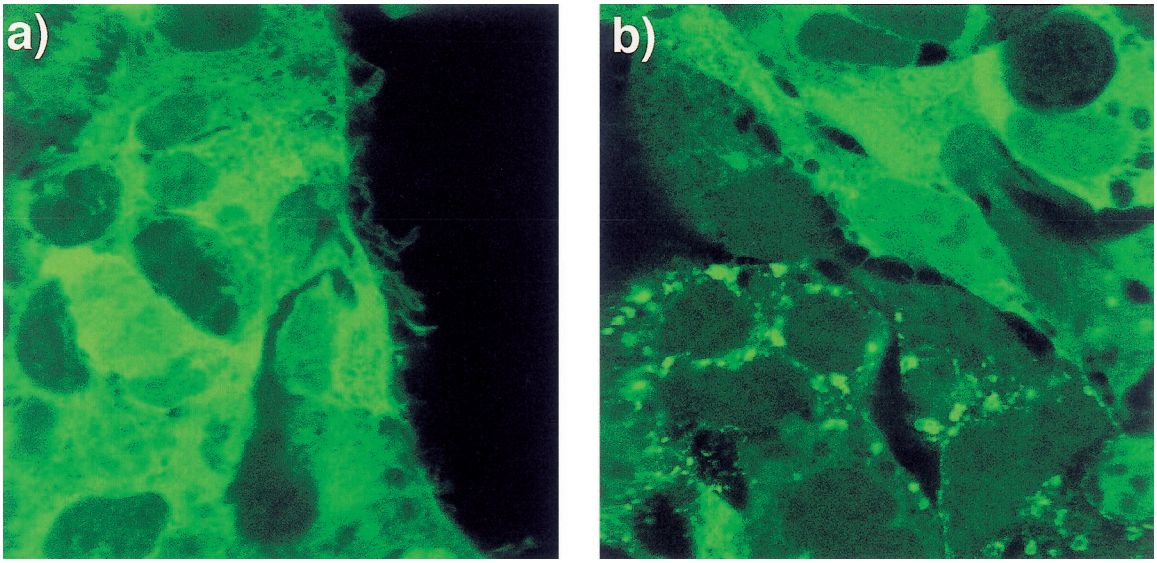

Figure 3 Cellular redistribution of β-arrestin-1-GFP in response to activation of the TRH receptor-1. β-arrestin-1-GFP (Groarke *et al*., 1999) was expressed stably in HEK293 cells. The long isoform of the rat TRHR-1 was then introduced transiently. In the absence of TRH distribution of β-arrestin-1-GFP was entirely even and cytoplasmic (a). Following treatment with TRH (10 μM, 30 min) the distribution of a fraction of the β-arrestin-1-GFP became punctate but only in a subset of the cells (b), whereas it remained evenly distributed throughout the cytoplasm of other cells. Further studies confirmed that the redistribution of β-arrestin-1-GFP was only obtained in cells which had positively expressed the TRHR-1 (see Groarke *et al*., 1999 for details).

indicated their co-localization. Following longer term incubation with TRH, a proportion of the β-arrestin 1-GFP could be seen to compartmentalize into large, punctate intracellular vesicles. This only occurred in cells which could also be shown to be immunopositive for the presence of the TRHR-1 and not in cells which were not transiently expressing the GPCR. Merging of internal GPCR and β-arrestin 1-GFP indicated them to be co-localized. Equivalent results were obtained by reversing the experimental protocol. Introduction and immunodetection of β-arrestin 1 in HEK293 cells stably expressing a TRHR-1–GFP construct initially demonstrated entirely separate locations of these proteins with the β-arrestin 1 distributed uniformly throughout the cytoplasm. Treatment with TRH (10 μM, 30 min) resulted in internalization of TRHR-1–GFP, a punctate appearance of a fraction of the cellular β-arrestin 1 and their internal co-localization as assessed by merging of the colour signals from the individual proteins (Groarke *et al*., 1999). Highly similar approaches have also been used to examine interactions and co-internalization of β-arrestin 1-GFP with both the neurokinin NK1 receptor (McConalogue *et al*., 1999) and the protease-activated receptor-2 (Dery *et al*., 1999). The capacity to observe apparent co-localization of internalized GPCRs and β-arrestins seems to be GPCR dependent, although a very wide range of GPCRs are able to promote recruitment of β-arrestins to the plasma membrane. For a number of GPCRs, including the β_2-adrenoceptor and the dopamine D_{1A} receptor, the β-arrestin recruited to the plasma membrane appears to remain there rather than being trafficked into the interior of the cells along with the GPCR. By contrast GPCRs such as the angiotensin II type 1A receptor, the neurotensin receptor (Zhang *et al*., 1999) and, as noted above, the TRHR-1 (Groarke *et al*., 1999) allow relocalization of the β-arrestins into intracellular vesicles in which they appear to be co-localized with the GPCR. Perhaps not surprisingly, the identity of the GPCR C-terminal tail plays a central role in whether the β-arrestin is co-internalized with the GPCR. Addition of the C-terminal tail of the angiotensin II type 1A receptor to the body of the β_2-adrenoceptor results in regulation of the cellular distribution of β-arrestins in a manner akin to that seen for the full-length angiotensin II type 1A receptor. Furthermore, the inverse pattern was produced with the complementary GPCR chimera (Zhang *et al*., 1999). Further work is likely to determine the detailed basis for these observations.

Agonist-induced translocation of transiently expressed β-arrestin 2-GFP to the plasma membrane has also been examined in HEK293 cells following co-expression with wild type and mutant forms of the hamster α_{1b}-adrenoceptor (Mhaouty-Kodja *et al*., 1999). Adrenaline (20 μM, 10 min) produced a clear translocation when added to cells expressing the wild type GPCR. Following expression of a constitutively active ($Ala^{293}Glu$) mutant of this GPCR a considerable amount of β-arrestin 2-GFP could be observed to be present at the plasma membrane without addition of agonist. This mutant also showed greater levels of agonist-independent phosphorylation than the wild-type GPCR. However, and compatible with the observation that addition of adrenaline can further stimulate the levels of phosphorylation of this mutant GPCR, addition of adrenaline resulted in a more pronounced translocation of β-arrestin 2-GFP (Mhaouty-Kodja *et al*., 1999). The generation of constitutive activity in terms of second messenger regulation does not necessarily correlate with enhanced GPCR phosphorylation. An Asp^{142}-Ala mutant of the hamster α_{1b}-adrenoceptor also displays a constitutive capacity to stimulate [^{3}H]inositol phosphate generation. However, this mutant was not effectively phosphorylated in either the absence or presence of adrenaline. Plasma membrane concentration of β-arrestin 2-GFP was not observed in the absence or presence of adrenaline following co-expression with this GPCR in HEK293 cells (Mhaouty-Kodja *et al*., 1999). It is also noteworthy that the Asp^{142}Ala form of the hamster α_{1b}-adrenoceptor is poorly internalized in response to adrenaline.

The differential capacity of morphine and etorphine to cause internalization of the μ-opioid receptor has for some time represented a pharmacological puzzle which has been assumed to relate to ligand efficacy. This issue has also recently been re-explored using β-arrestin 2-GFP translocation as an

assay end point (Zhang *et al*., 1998b). Transient transfection of HEK293 cells with the μ-opioid receptor allowed excellent internalization in response to etorphine but not morphine. This difference could not be overcome by co-expression of β-arrestin 1 but was when GRK2 was transiently introduced either with or without extra β-arrestin 1. In the absence of additional GRK2, although etorphine produced significant phosphorylation of the μ-opioid receptor, morphine did not. By contrast, in the presence of excess GRK2 both ligands were capable of producing strong phosphorylation of the GPCR, and indeed the extent of phosphorylation by either ligand was now substantially greater than produced by etorphine when extra GRK2 was not co-expressed. Etorphine-induced translocation of co-expressed β-arrestin 2-GFP could be observed without GRK2 expression, but it was weak and greatly enhanced by the presence of excess GRK2. Furthermore, a marked translocation of β-arrestin 2-GFP was produced by morphine only upon co-expression with GRK2 (Zhang *et al*., 1998b). Such results offer a plausible rationale for long observed differences in the desensitization potential of ligands at the μ-opioid receptor and suggest that varying expression levels of GRKs in different cells and tissues may be just as important in practical terms as the 'intrinsic activity' of the ligand in inducing differential patterns of GPCR desensitization to a ligand. It is thus of potential major significance that GRK levels are regulated in various disease processes including heart failure (Rockmann *et al*., 1998; Iaccarino *et al*., 1998, 1999) and hypertension (Gros *et al*., 1997).

Future directions

Simple application of the types of strategies discussed above, using a wide range of GFP-tagged signalling polypeptides, coupled with detailed pharmacological and biochemical experiments is likely to produce novel insights into a wide range of important issues in pharmacology. Although beyond the scope of this review, GFP-tagged forms of ion channel subunits, growth factor receptors with intrinsic tyrosine kinase activity, as well as many proteins involved in information transfer from the cell surface to the nucleus have already been generated and studied.

In terms of GPCR function and regulation two obvious and rapidly emerging strategies are likely to be widely employed. Mutant forms of GFP which have enhanced the brightness of the parent polypeptide have been central to the use of GFP tagging and imaging in live cells. A range of mutants which have resulted in altered spectral peaks for excitation and emission have already been characterized and the use of these in conjunction with the enhanced GFPs offers the possibility of differential tagging of two (or more) signalling proteins which can then allow concurrent dual imaging of the individual proteins in living cells (Ellenberg *et al*., 1999). This would represent a distinct advance over the combined use of GFP fluorescence and immunofluorescence which currently represents the cutting edge in the GPCR field. Such studies may find particular application in studying the dynamics of dephosphorylation of GPCRs and their recycling to the plasma membrane with concurrent examination of the consequences for cellular β-arrestin distribution. Cloning of other bioluminescent proteins from a variety of marine species is also likely to expand the chromogenic palette available for imaging studies.

There has also been considerable interest in the application of fluorescence resonance energy transfer (FRET) techniques to the interaction of proteins within signalling cascades (Pollock & Heim, 1999). Given that spectrally modified forms of GFP have been identified which have the capacity to act as FRET partners, then as the basis of the technique requires that two partners are brought into (or are removed from) close physical proximity, it may prove a highly appropriate way to examine the issue of GPCR dimerization and potential regulation of such dimers by agonist ligands (Hebert *et al*., 1996; Cvejic & Devi, 1997). A potentially even more useful alternative to FRET is bioluminescence resonance energy transfer (BRET) which can be achieved between a polypeptide linked to a GFP-variant and one linked to an appropriate luciferase (Xu *et al*., 1999). Assuming that attachment of luciferase (or other similar reporters which provide a luminescence output) to a GPCR does not interfere with the basic function and pharmacology of the protein it should be possible to use BRET, which produces a clear bioluminescent signal in the region of 520–550 nm to directly report the types of protein–protein interactions discussed in the review.

Undoubtedly further technical developments are to come but it is already clear that imaging and measuring real time pharmacological processes initiated by ligand binding to a GPCR has a very 'bright' future.

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