ASSAY and Drug Development Technologies
Volume 6, Number 5, 2008

DOI: 10.1089/adt.2008.160

Technology Review

Photoproteins: Important New Tools in Drug Discovery

Richard M. Eglen[1] and Terry Reisine[2]

Abstract: The G protein-coupled receptor (GPCR) family is a major target for drug discovery, and most, if not all, GPCRs can couple to Ca^{2+} signaling. Consequently, there are a number of cell-based, primary, high-throughput screening (HTS) assays used for drug discovery that assess changes in intracellular Ca^{2+} as a functional readout of GPCR activation. Historically, changes in intracellular Ca^{2+} levels have been readily detected using fluorescent dyes that emit light in proportion to changes in intracellular Ca^{2+} concentration. An alternative approach to indirectly measure changes in Ca^{2+} concentrations involves the use of recombinantly expressed biosensor photoproteins, of which aequorin is a prototypic example. These biosensors have the advantage that they provide an intense luminescent signal in response to elevations in intracellular Ca^{2+}. This exquisite sensitivity, the high signal-to-noise ratios, and the ability to target expression to discrete subcellular sites (in order to detect Ca^{2+} microdomains) have made photoproteins a principal choice in a wide range of GPCR drug discovery programs. Photoproteins are also finding increasing use in detecting activation of other molecular target classes such as ligand-gated ion channels and transporters. This review focuses upon the use of calcium photoproteins principally for use in GPCR drug discovery and HTS.

Introduction

Due to their role in cell–cell communication and in the etiology of many diseases, G protein-coupled receptors (GPCRs) continue to be prime targets for drug discovery.[1,2] GPCRs localize to the cell membrane where they bind a wide and diverse range of ligands, including neurotransmitters, hormones, growth factors, synthetic compounds, and other molecules that affect intracellular signaling cascades.[3] The intracellular domains of GPCRs associate with heterotrimeric G proteins, which serve to translate the conformation changes in the ligand-bound GPCRs to a discrete cellular response. Since activation of the cellular pathways provides a degree of signal amplification, many assays used in GPCR HTS employ cell-based ("functional") approaches.[4] The assays generally measure changes in second messenger accumulation consequent to ligand binding and GPCR activation. Although GPCRs and their cognate G protein(s) naturally affect signaling via several second messengers, and in many cases a single GPCR modulates multiple second messengers simultaneously,[5–8] one second messenger system widely used as a general readout for GPCR activation involves measurement of changes in intracellular Ca^{2+} concentration.

Ca^{2+} ions are critical for many cellular functions, being particularly important in modulating the activity of enzymes involved in signal transduction. The levels of free cellular Ca^{2+} (that is, Ca^{2+} unsequestered to proteins or other organelles) is modulated via multiple mech-

[1]Bio-discovery, PerkinElmer Life and Analytical Sciences, Waltham, MA.
[2]Independent consultant.

ABBREVIATIONS: CCD, charge-coupled device; DMD, Duchenne muscular dystrophy; FLIPR™, fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA); GFP, green fluorescent protein; GPCR, G protein-coupled receptor; IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; PLC, phospholipase C.

anisms, including influx from extracellular sites via ionic conductance channels, efflux by way of channels, transporters, and various energy-dependent pumps, and through movement from storage sites that sequester free cytosolic Ca^{2+} such as the endoplasmic reticulum.[9] These diverse pathways also suggest that measurement of Ca^{2+} changes provides an approach to screen for other targets, including calcium channels and transporter proteins.

GPCR Regulation of Intracellular Ca^{2+} Concentration

GPCR activation modulates cytosolic free Ca^{2+} via association with different G proteins. For example, several GPCRs regulate Ca^{2+} influx through voltage-sensitive ionic conductance channels via the G protein G_o.[10–13] Specifically, GPCR stimulation causes dissociation of the β/γ subunits from the α subunit of the heterotrimeric G_o complex, and the free β/γ subunit acts upon voltage-sensitive Ca^{2+} channels to modulate Ca^{2+} transport through the plasma membrane. This process is particularly important for GPCRs in central nervous system neurons, especially as G_o is the predominant G protein expressed in brain.[14] Moreover, many GPCRs modulate neuronal activity by coupling to G_o. GPCRs can also modulate levels of cytosolic Ca^{2+} in cells by activating phospholipase C (PLC) and phosphatidylinositol (PI) signaling. $G_{q/11}$ proteins are the predominant G proteins mediating GPCR regulation of Ca^{2+} stores, and, as may be expected, several GPCRs couple to both G_o and $G_{q/11}$ to regulate cytosolic Ca^{2+} levels through either mechanism.[1]

In addition to $G_{q/11}$, the G proteins G_{16} and its murine ortholog G_{15} also couple activation of GPCRs to changes in free cytosolic Ca^{2+}.[15] These G proteins are unusual in that they associate with many different types of GPCRs.[16] Such promiscuity has resulted in their use in generic assays of GPCR function. Specifically, by expressing $G_{15/16}$ in cells, one can artificially link ("force couple") most GPCRs to PI signaling, thereby providing a simple and generic assay for GPCR activation. This has been shown with GPCRs that normally couple to PI signaling and those that are not known to couple to this signaling, as well as orphan GPCRs for which little or nothing is known about their endogenous cellular responses. This approach is also useful irrespective of the natural G protein composition in the target cell under study because by overexpressing $G_{15/16}$ in cells, those G proteins become the obvious G protein linked to the receptor and dominate over any other potential GPCR/G protein linkage.

Additional approaches to develop universal GPCR signaling assays for drug discovery include approaches to facilitate coupling of G_i-linked receptors to PLC and PI signaling with chimeric G_q proteins such as G_{qi5}. This chimeric G_q protein has the C-terminal five amino acids of G_i instead of the natural G_q sequence. This manipulation appears to facilitate the ability of Gi-coupled receptors to stimulate PLC[17,18] since the C-terminal five amino acids of the α subunit are critical for coupling to GPCRs, thereby allowing the mutant G_q to couple to a receptor that normally would not associate with wild-type G_q. Thus, use of either $G_{15/16}$ or G_{qi5} stably expressed in cells is believed by some to provide a universal GPCR assay allowing almost any GPCR to be linked to PLC, providing an assay now widely used in drug discovery.

Functional Assays of GPCR Activation

Automated cell-based assays to measure GPCR activation have been developed for HTS assays in drug discovery and have used a range of different technologies focused on different components of receptor stimulation. In general, they can involve use of fluorescently labeled ligands to detect directly agonist or antagonist GPCR binding, fluorescence resonance energy transfer, bioluminescence resonance energy transfer, and protein complementation techniques to detect GPCR association with G proteins or other cellular regulators including β-arrestin, measurement of second messengers such as cyclic AMP, or detection of protein kinase activity such as detection of extracellular signal regulated kinase $^1/_2$.[1] However, one technology widely employed by many screening labs involves measurement of changes in intracellular Ca^{2+} levels as a functional marker of GPCR activation.

Changes in intracellular Ca^{2+} levels can be detected directly using electrophysiological techniques that monitor changes in Ca^{2+} conductance. However, this approach is not readily adaptable for HTS, although recent advances in automated patch clamp technologies or multiple electrode chip electrophysiology to record from multiple cells[19–23] make this approach feasible in secondary screening. Classically, therefore, the principal technology to measure cytosolic Ca^{2+} levels employs probes whose fluorescent response changes in the presence of Ca^{2+} upon laser excitation. The probes are low-molecular-weight dyes, including Fura-2, Fluo-4, Fluo-8, and Calcium 3. When these dyes chelate Ca^{2+} they emit a fluorescent response, the intensity of which is directly proportional to the concentration of free Ca^{2+} levels.[24–27]

A major advance in the last decade or so involves use of photoprotein biosensors to detect free intracellular Ca^{2+} ions.[9,28,29] By using recombinant approaches, photoprotein can be transiently or stably expressed in a range of cell phenotypes. They emit an intense luminescent signal in the presence of free Ca^{2+} in the absence of a light stimulation, which reduces background signals. Since

they are recombinant proteins, one can, by using appropriate targeting sequences, localize the biosensor to specific cellular compartments, such as the mitochondria or cell membrane, to monitor changes in Ca^{2+} levels in specific subcellular regions, providing further advantages over dyes to monitor Ca^{2+} levels.

Fluorescent Calcium Dyes to Measure Changes in Intracellular Calcium Concentration

Ca^{2+}-chelating dyes such as Quin-2 and Fura-2 were used over 2 decades ago to measure Ca^{2+} transients in cells in response to GPCR activation.[24,30,31] The acetoxymethyl ester forms of these dyes are cell permeant and thus freely enter the cell following a suitably long incubation period. Once in the cell they are de-esterified to a form that is less permeable and are thus retained for the period of the experiment. In the presence of Ca^{2+}, the emission wavelength of the dye changes, providing a means to detect changes in intracellular Ca^{2+} levels. Thus, a light source is used to excite the dye in cells, and the difference in fluorescent emission when the dye is bound or not bound to Ca^{2+} reflects changes in intracellular Ca^{2+} levels. This simple procedure to measure Ca^{2+} transients has improved with both the development of newer dyes, such as Fluo-4 and Calcium 3,[32,33] and Fluo-8 automated "inject and read" detection systems such as the fluorometric imaging plate reader (FLIPR™, Molecular Devices, Sunnyvale, CA).[34–36] Finally, the implementation of procedures that obviate extensive cellular washing to remove extracellular dye has increased the both viability of the dye-loaded cells and diminished cellular stress, both of which induce an artifactual excitation of the cell.[32,37]

Although fluorescent dyes have been used extensively in GPCR HTS there are some limitations to the approach (Table 1). One of these relates to the relatively low sensitivity of the assay stemming from cellular background fluorescence of dye-loaded cells.[38] As a result, the levels of Ca^{2+} transients following GPCR activation is not as robust as one would desire, particularly with those GPCRs poorly coupled to PLC and PI turnover. Although this may not be such a problem when identifying GPCR agonists, it creates difficulties in identifying partial agonists, inverse agonists, and antagonists for which small responses must be discerned against high background signals. A second, frequently encountered problem is that numerous compounds in small molecule libraries affect the fluorescent signal from these Ca^{2+}-sensitive dyes via quenching or autofluoresence, resulting in spurious "false-positive or -negative hits" being identified in a primary screen.[39] Although this can be averted by prescreening compounds, when libraries of millions of compounds are screened, such activities are both time consuming and expensive.

Collectively, these deficits have lead to the development in the use of a family of sensitive photoproteins, such as aequorin, that emit bioluminescent signals when changes in intracellular Ca^{2+} occur (see Table 1 for comparison with fluorescent dyes).[28,29,38,40–42] A major difference in the use of dyes versus photoproteins is due to the inherent differences associated with fluorescence versus chemi- or bioluminescence. In fluorescence, the excitation of the fluorophore is dependent on light, whereas for bioluminescence, an exothermic chemical reaction is

Table 1. Comparison of Ca^{2+}-Sensitive Dyes and Photoproteins for HTS

Properties	*Ca^{2+}-sensitive dyes*[a]	*Photoproteins*[b]	*GFP sensors*[c]
Energy emission	Fluorescence	Chemiluminescence	Fluorescence
Detection device	FLIPR	FLIPR, CCD	Microscopy
Backgrouns Ca^{2+}	Moderate to high	Low	Moderate/high
Signal-to-noise ratio	Low to moderate	High	Moderate
Dynamic range of Ca^{2+} change	Low to moderate	High	High
Sensitivity	Low to moderate	High	High
Adaptability to assay conditions	Low	Moderate/high	Low
Cells per well needed	Thousands	Hundreds	Single/hundreds
Ease of use in HTS	High	High	Moderate
Compount interference	Moderate	None	Moderate
Useful for orphan GPCRs	Not easy	Yes	Moderate
Targeted to subcellular sites	No	Yes	Yes
Ca^{2+} in microdomains	No	Yes	Yes
Reaction kinetics	Fast	Photina slow	Fast
Used *in vivo*	No	Yes but rarely	Yes

[a]Includes the dyes Fura-2, Fluo-4, Fluo-8, and Calcium 3.
[b]Includes aequorin and Photina.
[c]Includes cameleons, camgaroos, and pericams.

necessary for excitation. As a consequence, in fluorescence measurements there is higher background light, whereas in chemiluminescence there is no background light. This results in very high signal-to-noise ratios for chemiluminescent reactions, whereas fluorescent responses are usually plagued by lower ratios and greater basal backgrounds.

Another advantage of the bioluminescent responses is that they have a high quantal efficiency such that few photons need to be emitted in order to be detected by sensitive charge-coupled devices (CCD), now standard automated detection systems routinely used in GPCR drug discovery. Because of the greatly sensitive response, relatively few bioluminescent photoprotein emitters are needed to be expressed per cell for detection. Indeed, the photoproteins can be expressed at attomolar levels in cells in order to detect intracellular Ca^{2+} changes using CCD or photomultiplier devices.[29] This provides major advantages over fluorescent dyes since fewer cells need to used in the assay, and miniaturization to very small fluid volumes (as would be used in 1,536-well microtiter plate assays) becomes feasible. The large signal-to-background ratios of the luminescent assays allows for robust detection of weak partial agonists or positive allosteric modulators of the GPCR in a primary HTS mode, both of which cannot be easily seen with responses having high background and low dynamic range of response. Finally, photoproteins have much lower Ca^{2+} buffering ability than the chelating calcium dyes, thereby producing less sequestration of intracellular Ca^{2+}. Consequently, there is less perturbation of intracellular Ca^{2+}, which improves cell function and viability. Taken together, most new approaches for GPCR drug discovery now employ photoprotein biosensors.

Photoproteins to Measure Changes in Intracellular Calcium Concentration

Several photoproteins have been isolated and cloned, although the one most widely used for measuring GPCR-induced Ca^{2+} transients is aequorin, originally isolated from jellyfish.[29,40,43,44] This protein has a central hydrophobic core that binds the imidazopyrazinone chromophore, coelenterazine.[45,46] (Figure 1 gives the structure of coelenterazine and the crystal structure of aequorin.[47]) Ca^{2+} ion binding to aequorin induces oxidation of coelenterazine to the excited form, coelenteramide (Fig. 2 shows the reaction). Relaxation from the excited state results in blue light emission at 465 nm, which can be detected using either a photomultiplier tube or CCD camera. There is generally low background luminescent emission from most cells used for drug discovery, and in those cells expressing aequorin there is little background bioluminescence unless aequorin has access to increased Ca^{2+}. Consequently, the flash luminescence associated with aequorin provides a very high signal-to-noise response ratio. More improvements have been made in aequorin through site-directed mutagenesis[47] and the coelenterazine structure[40] to further enhance the kinetics of coelenterazine decay from apoaequorin. This provides different forms of light emissions to reduce the decay, thereby prolonging the bioluminescent signal and enhancing its utility as a biosensor in a wide range of detection instruments.

An important advantage of aequorin over fluorescent dyes is that its cloning allows the protein to be expressed stably in cell lines, thereby providing a consistent source of cells expressing the biosensor. One then can optimize GPCR signaling detected by aequorin bioluminescence by recombinantly manipulating the appropriate levels of receptor, G protein, and aequorin expressed in the cell. Just as important is that, using targeting sequences, aequorin can be selectively expressed in specific organelles to measure microdomains of Ca^{2+} signaling, something that cannot be done easily with dyes (Table 1).[48–52] Indeed, targeting aequorin to the mitochondria provides a more accurate and robust measure of GPCR-stimulated Ca^{2+} transients than expression throughout the cytoplasm given that the mitochondria are spatially close to Ca^{2+} channels and release sites from the endoplasmic reticulum.[9,52] Thus, aequorin targeted to those sites is better at monitoring the rapid increases in Ca^{2+} levels following GPCR/G_q/PI signaling than if the photoprotein is diffusely expressed throughout the cytoplasm, where its detection of Ca^{2+} may be distal from the site of generation of Ca^{2+} transients.

The high sensitivity and low background of aequorin compared to dyes for measuring GPCR signaling are a major advantage in identify novel ligands such as ligands binding to orphan GPCRs. When searching for the ligand of an orphan receptor, no reference ligand is available to allow the optimization of its coupling to the calcium pathway prior to performing the screening, resulting in nonoptimal coupling to calcium in the cells used for screening. When background luminescence is relatively high, because Ca^{2+} levels are measured throughout the cell and not just at the site of Ca^{2+} release from storage sites or channel entry points, as in the case of the use of dyes, the ability to discriminate small increases in intracellular Ca^{2+} induced by low agonist concentrations or in situations where calcium coupling cannot be optimized is hindered. In contrast, when photoproteins such as aequorin are targeted to sites that primarily measure Ca^{2+} release and when basal bioluminescent readout is very low or nonexistent, then the relatively small decreases in Ca^{2+} transients can be detected. Low background and high sensitivity of the aequorin assay are real advantages to allow detection of even small agonistic activities under these challenging screening conditions. Photoprotein sensitivity is also an advantage when screening deorphanized receptors. For example, because of the high sensitivity of

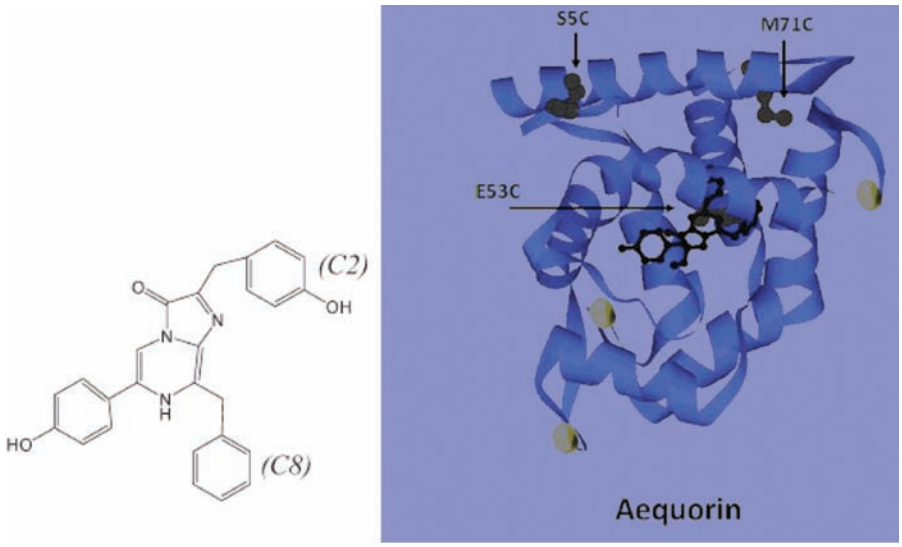

FIG. 1. Structure of colenterazine (**left**) and crystal structure of aequorin (**right**). Modified from Rowe *et al.*[47]

the aequorin assay, low reference agonist concentrations may be used when setting up an antagonist screen, while maintaining a good assay window and statistical parameters. This places the aequorin assay in good position to detect less potent inhibitors.

Subcellular Targeting of Aequorin to Measure Microdomain Ca^{2+} Transients

In addition to mitochondrial aequorin targeting, the photoprotein can also be selectively expressed in other organelles, such as the plasma membrane,[53,54] endoplasmic reticulum,[55] nucleus,[56–58] and secretory vesicles.[59,60] Using targeted aequorin expression, it has been reported that not only do Ca^{2+} transients differ in one microdomain versus another, but also that compartmental Ca^{2+} transients vary with GPCR stimulation.

Photoproteins in plasma membrane

Targeting aequorin to the inner surface of the plasma membrane, using either the membrane protein SNAP-25 or adenylyl cyclase, allows measurement of Ca^{2+} levels at the subplasma membrane level.[9,53,61] It has been found that cellular activation leads to augmented Ca^{2+} transients compared with Ca^{2+} transients generated in the

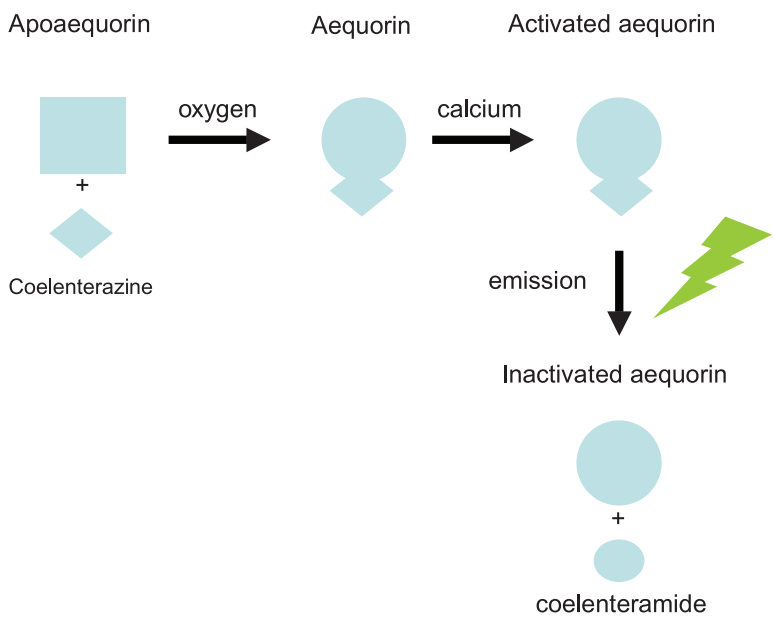

FIG. 2. Aequorin reaction. The ability of aequorin to detect Ca^{2+} intracellularly is based a defined chemical reaction. Apoaequorin binds the cofactor colenterazine to generate aequorin. Ca^{2+} binds to aequorin, resulting in oxidation of coelenterazine, which gives off a blue light emission at 471 nm. Once the Ca^{2+} binds, the aequorin is essentially inactivated and cannot be used to further detect Ca^{2+}.

bulk cytoplasm. This finding was confirmed using probes such as the green fluorescent protein (GFP)-based Ca^{2+} probes, the cameleons.[9] Ca^{2+} ion changes at the subplasma membrane level are believed to arise primarily, although not exclusively, from influx via voltage-dependent cell membrane Ca^{2+} channels. These membrane-localized, large increases in Ca^{2+} transients are regulated in response to neurotransmitter receptor activation and may be related to "hot spots" found in neurons and endocrine cells in the vicinity of Ca^{2+} channels. Moreover, these rapid and robust changes have also been linked to synaptic vesicle fusion and eventual neurotransmitter release. This phenomenom is particularly important since synaptic vesicle release requires more robust increases in Ca^{2+} transients than in those generally found in the cytoplasm as a whole. Since the activity of many voltage-dependent Ca^{2+} channels is endogenously regulated by ligands acting via G_o-coupled GPCRs,[10–13] measurement of plasma membrane Ca^{2+} transients provides a direct reflection of GPCR stimulation via G_o in comparison to those linked to $G_{q/11}$, which predominantly affect Ca^{2+} transients via PLC/PI turnover mechanism and consequently Ca^{2+} release from the endoplasmic reticulum. However, this interpretation is only valid if the plasma membrane-targeted aequorin does not detect Ca^{2+} transients via PLC/PI turnover since the endoplasmic reticulum, which releases Ca^{2+} via a PI mechanism, can be in close proximity to the plasma membrane and those GPCRs that couple to G_o and voltage-dependent Ca^{2+} channels can also modulate PI turnover.

Large changes in Ca^{2+} transients at plasma membrane microdomains are particularly important for several GPCR-regulated signaling pathways. Thus, several reports have shown that the changes in Ca^{2+} in the vicinity of the voltage-dependent Ca^{2+} channels are critical to regulate mitogen-activated protein kinase kinase pathways and in inducing the phosphorylation and activation of CREB.[9] Both pathways are involved in mediating both short-term cellular effects of GPCR activation as well as longer-term effects such as changes in gene transcription. Furthermore, changes in subplasma membrane Ca^{2+} appear to oscillate closely with changes in Ca^{2+} levels in paranuclear regions measured using targeted photoproteins, suggesting that patterns of Ca^{2+} signaling at the plasma membrane affect several distal events in the cell.[9]

These findings in general indicate that using plasma membrane-bound aequorin is useful in detecting GPCRs linked to changes in plasma membrane potential and influx of Ca^{2+} via voltage-dependent Ca^{2+} channels, as opposed to those GPCRs primarily coupled to inositol 1,4,5-trisphosphate (IP_3) production and PI turnover (which regulates Ca^{2+} release from endoplasmic reticulum stores). As these different pathways require receptor coupling to distinct G proteins, the use of plasma membrane-bound aequorin versus aequorin expressed in the endoplasmic reticulum or mitochondria provides a screening technology to selectively identify drugs targeting GPCRs controlling Ca^{2+} levels in different microdomains. In effect, by using these different Ca^{2+} readouts, it may be possible to identify novel compounds capable of acting on the same GPCR to "channel" different biological pathways.

This approach is useful since many cells do not express a full complement of G proteins. Thus, several cells express little, if any, G_o. Indeed, many of the cell lines used in HTS, such as human embryonic kidne 293 cells, do not express G_o.[62–64] Consequently, in human cells expressing low levels of G_o, GPCR signaling is most predominantly coupled to G_q with subsequent signaling occurring via release of intracellular Ca^{2+}. Other cells, such as neurons, predominantly express G_o with relatively little G_q, and low levels of G_i and GPCR signaling may thus occur primarily via regulation of voltage-sensitive Ca^{2+} channels.[14] Recent studies have shown that different G proteins affect the ligand binding properties of receptors, and as a consequence, depending on the G proteins expressed in the cell, the pharmacological properties of the GPCR may differ from cell to cell.[62,65,66] It is possible that one could develop drugs that selectively modulate GPCRs coupled to G_o differently from the same GPCR coupled to G_q, *i.e.*, by the use of techniques that discriminate GPCR/G_o from GPCR/G_q signaling. Assays distinguishing these signaling pathways may conceivably be used to identify designer drugs that discriminate these distinct receptor complexes to produce different cellular responses via the same GPCR.

The approach of targeting aequorin to the plasma membrane has already been shown to have several potential uses in drug discovery. An example is found in studies by Basset *et al*.,[53] who investigated Ca^{2+} signaling in myotubes from a mouse genetic model of Duchenne muscular dystrophy (DMD), a disease in which gradual degeneration of muscle cells leads to death due to respiratory and cardiac failure. Muscle cells in DMD have altered Ca^{2+} homeostasis because of loss of dystrophin. This is believed to lead to muscle degeneration. In DMD myotubes, Ca^{2+} influx and levels at the subplasma membrane are greatly elevated. Basset *et al*.[53] expressed aequorin targeted to the plasma membrane of control and DMD myotubes and showed that stimulation of nicotinic receptors in the cell membrane of the DMD muscle cells resulted in a greatly exaggerated rise in cellular Ca^{2+} rather than Ca^{2+} transients in normal muscle cells. In the control myotubes the rise in cell membrane Ca^{2+} was almost exclusively due to membrane depolarization and Ca^{2+} influx via voltage-sensitive Ca^{2+} channels. In the DMD myotubes, IP_3-mediated release of Ca^{2+} stores contributed to the exaggerated Ca^{2+} response. However, receptor stimulation was not involved in nicotinic-induced Ca^{2+} response in control myotubes. Thus, in

DMD, an abnormality in Ca^{2+} signaling in muscle cells occurs resulting from the involvement of IP_3 signaling and stimulation of Ca^{2+} mobilization that caused much more Ca^{2+} to be available in the cell than normal, which leads to dysfunction and death.

This distinction raises the possibility of developing compounds that selectively target IP_3-mediated Ca^{2+} signaling in myotubes as an approach to treat the disease. Such an approach could involve identifying GPCRs expressed in muscle cells and linked to G_q, which could be the target for identifying inverse agonists (drugs that would reduce IP_3-mediated Ca^{2+} transients), which would diminish the exaggerated Ca^{2+} signaling in the disease cells. This is one example of using photoproteins targeted to different compartments to identify unique disease-related events and then using those unique properties to discover disease-selective drugs to treat the disorder.

Photoproteins in mitochondria

Aequorin and other photoproteins have also been targeted to mitochondria, using cytochrome c oxidase targeting sequences.[48–52] The mitochondrion is an important organelle in that it is the major site of energy production in the cell. It takes up Ca^{2+} via a specific antiporter, which generally has relatively low affinity for Ca^{2+}. Numerous studies have indicated that targeting aequorin and other photoproteins to the mitochondria provides a more robust measure of GPCR-regulated Ca^{2+} transients than simply expressing the sensor in the cytoplasm. This is likely due to the localization of mitochondria in close proximity of IP_3-gated Ca^{2+} channels in the endoplasmic reticulum. This hypothesis has been further supported by electron microscopic analysis using GFP tags of mitochondria and endoplasmic reticulum showing close opposition of mitochondria to Ca^{2+} release sites on the endoplasmic reticulum.[9] Thus, stimulation of the release of Ca^{2+} stores following stimulation of GPCRs linked to PLC and IP_3 signaling produces a spike in the level of Ca^{2+} near mitochondria, more intense than generally seen in the cytoplasm, and this rapid rise in Ca^{2+} may explain how sufficient levels of Ca^{2+} can enter mitochondria to regulate activity despite the low affinity of the antiporter for this ion.

From an assay perspective, targeting aequorin to the mitochondria to detect Ca^{2+} transients provides a selective recording of GPCR/G_q-mediated signaling since much of the rise in mitochondria Ca^{2+} uptake is due to release of Ca^{2+} from endoplasmic reticulum stores—which is regulated by an IP_3-mediated mechanism. This is supported by studies of Montero *et al.*,[55] who targeted aequorin to the lumen of the endoplasmic reticulum and showed the Ca^{2+} release from this organelle was almost exclusively due to an GPCR agonist-induced IP_3-mediated mechanism, and since the Ca^{2+} released is in close proximity to the mitochondria, aequorin monitoring of Ca^{2+} levels near the mitochondria provides a good measure of GPCR/IP_3 signaling.

Photina®

Historically, aequorin has been used extensively to study Ca^{2+} signaling both in basic research and in drug discovery, but it has characteristics that can be improved upon. Specifically, it has relatively weak energy emission and rapid kinetics. These properties diminish its utility in cell-based assays. The low emission requires more cells to be used per assay, and the rapid kinetics affect assay throughput and diminishes versatility.

To overcome these problems, Bovolenta *et al.*[38] have developed a new chimeric photoprotein designated as Photina (Axxam, Milan, Italy). This is a mixture of two naturally occurring photoproteins, obelin and clytin. Obelin, like aequorin, is a weak energy emitter. However, its reaction kinetics with Ca^{2+} and coelenterazine are slower than aequorin, providing a longer readout of the luminescent response.[67] Clytin is a photoprotein with poor Ca^{2+} sensitivity compared to aequorin, but some forms of clytin have recently been shown to have larger luminescent intensity than aequorin.[68,69] In order to combine the attributes of both proteins, Bovolenta *et al.*[38] exchanged a fragment of clytin into the vicinity of the EF hand structure of obelin. The chimeric protein, Photina, can then be expressed in cells, just like aequorin, and be targeted to subcellular localization, such as the mitochondria and cell membrane, or be simply expressed throughout the cytoplasm.

Photina generated larger luminescent response than aequorin, yielding a threefold greater signal to GPCR-stimulated Ca^{2+} transients in cells than an aequorin biosensor.[38] It gives comparable pharmacological results in the FLIPR for GPCRs measured using calcium and Calcium-3 assay kits (fluorescent assays). Moreover, the sensitivity of Photina was about 10-fold greater than standard fluorescent calcium dyes used in FLIPR assays. In fact, the sensitivity of Photina was so high that as few as 100 cells per well could be used in screening assays.[38] This is an important advantage over aequorin and calcium dyes since it means that Photina can be used in ultra-HTS assay systems and has already been shown to work successfully in 1,536-well format. The Photina system can be detected in almost any device that measures light emission and is easily adapted to detection in various fluorescent systems such as FLIPR, which are not ideally employed for luminescent measurements, as well as more standard bioluminescent devices.

An important advantage of Photina over aequorin is the slower reaction kinetics. Aequorin rapidly gives off a luminescent signal (within 30 s), which hinders its use in standard HTS formats and requires the use of integrated application systems. Photina reaction kinetics are much more adapted to HTS systems and allow for detection of a greater range of Ca^{2+} kinetics. Furthermore, because of the greater strength of the luminescent response, Photina measurements can be used in fluorescent readers as well as luminescent readers, allowing a greater choice of systems to employ, whereas aequorin is primarily measured on bioluminescent readers. It is important that the slower kinetics of the Photina luminescent reaction allow for improved detection of Ca^{2+} kinetics so that extremely rapidly changes in intracellular Ca^{2+} as may occur with either GPCR-regulated or voltage-regulated ionic conductance channels can be detected and analyzed in HTS mode, whereas the rapid kinetics of the aequorin flash luminescence makes these kinetic measurements more difficult to detect. In fact, Photina appears to be adaptable for HTS of ionic conductance channels as well as GPCRs.

The major advantages of Photina over use of Ca^{2+} dyes are similar to those of aequorin. The lack of autoluminescent and extremely low background provide a greater dynamic range of measurement of Ca^{2+} responses and higher signal-to-noise ratios of Photina measurements than seen with standard fluorescent assays. Furthermore, with Photina, there is no interference of compound autofluorescence, which is a common drawback of fluorescent Ca^{2+} indicators. The high sensitivity of Photina also means many fewer cells are needed than in standard Fluo-3 or Fluo-4 assays, which can be a major advantage when screening millions of compounds in large screening campaigns. This is further compounded by the ability of targeting Photina to mitochondria, which provides an even more sensitive detection of subcellular Ca^{2+} release from storage sites than can be seen with dyes. Thus, Photina is an improved version of aequorin that in many ways is superior to use of other Ca^{2+} fluorescent indicators for HTS.

Cameleons, Camgaroos, and Pericams

In addition to aequorin and Photina, a second family of photoproteins has been developed to measure Ca^{2+} transients that are fluorescent based and that have been mainly employed for research purposes. This family consists of at least three types of biosensors referred to as cameleons, camgaroos, and pericams, all of which consist of mutant forms of GFP. Like the fluorescent dyes, these GFP-based Ca^{2+} sensors require light stimulation to evoke a fluorescent emission in contrast to aequorin and Photina, which emit bioluminescence as a consequence of a chemical reaction.

Cameleons consist of two mutant forms of GFP: a cyan-emitting mutant and an enhanced yellow-emitting mutant.[52,70–75] These two GFP forms are connected linearly by calmodulin, a linker, and the calmodulin binding domain of myosin light chain (M13). The sensor detects Ca^{2+} by its binding to the calmodulin component of this large fusion protein, which causes an interaction of calmodulin with M13 resulting in conformational changes that bring the two mutant forms of GFP in close proximity (in contrast to the Ca^{2+}-free form, which is extended with the two GFP mutants separated). When the two mutant GFPs are near each other, an energy transfer from the cyano to the yellow form occurs. The excitation wavelength of the cyano form is 458 nm, and emission is at 480 nm. As a result of the fluorescence resonance energy transfer reaction, the emission shifts from 480 nm to 530 nm as a function of increasing concentrations of Ca^{2+}. The cameleons give off a stronger light response than aequorin, do not require addition of factors such as coelenterazine, and regenerate activity when Ca^{2+} dissociates. This allows the cameleon to be used as a continuous biosensor of Ca^{2+} responses in cells. This provides an advantage over aqueorin or photina because in their case, the sequestration of Ca^{2+} while producing a bioluminescence signal also reduces the cytoplasmic biosensor concentration (each aequorin molecule that binds Ca^{2+} becomes inactive). Thus, aqueorin cannot be easily used for repeated *in vitro* measurements of Ca^{2+} signaling following multiple stimulations.

Like aequorin, cameleons can also be targeted to subcellular locations using selective targeting sequences to measure Ca^{2+} levels in selective compartments.[9,75] Furthermore, single amino acid mutations of the cameleons can be used to greatly change sensitivity of the biosensors. This allows one to generate cameleons with levels of sensitivity advantageous in measuring Ca^{2+} levels for the subcellular compartment under study. For example, very sensitive cameleons can be used to measure the very low resting Ca^{2+} levels inside the mitochondria. However, different probes can be made to provide optimal readouts of the high levels of Ca^{2+} on the outer membrane of the mitochondria, where greatly elevated Ca^{2+} levels would occur as a result of Ca^{2+} release from the endoplasmic reticulum. In later case, the highly sensitive probes would be rapidly saturated, reducing determination of the linearity of response.

Camgaroos are modified forms of cameleons in which a calmodulin molecule is inserted into a single cyclic GFP molecule in which the N- and C-termini are fused.[76,77] Ca^{2+} binding to the calmodulin produces a large increase in fluorescence emission from the GFP, and as with the cameleons the response is reversible. Pericams, like ca-

magroos, are circular GFP molecules in which calmodulin and M13 are inserted so that Ca^{2+} binding to calmodulin promotes interaction of calmodulin with M13, presumably producing greater conformational changes that alter the chromophore than with just calmodulin alone, to produce significant fluorescent emission.[72] Compared with cameleons, pericams produce much greater fluorescent responses to Ca^{2+} binding and have a better signal-to-noise ratio.[52] Furthermore, they have properties that distinguish them from camagroos in that they have much greater affinity for Ca^{2+}, making them better suited for measuring physiologically relevant, GPCR-regulated Ca^{2+} spikes in cells. The exquisite sensitivity of pericams allows them to measure Ca^{2+} transients in single mitochondria, making these sensors very useful for cell biological research.

In fact, the cameleons, camgaroos, and pericams have primarily been employed for basic cell biological studies, usually in single-cell analysis. For example, studies measuring "hot spots" of Ca^{2+} in the subplasma membrane region used cameleons differentially targeted to the caveolae (using caveolin-1)[78] and the plasma membrane in general (using a 20-amino acid-long, doubly palmitoylated, amino-terminal portion of neuromodulin/growth-associated protein 43) as well as a nontargeted cameleon that distributed throughout the cytoplasm.[9] Furthermore, because of the strong emissions from the cameleons it was possible to show, using fast-single cell imaging targeted to the mitochondria and other subcellular sites, that the GPCR-stimulated oscillations in cytosolic Ca^{2+} paralleled temporally the rapid spiking in Ca^{2+} detected near the mitochondria, consistent with the idea that activation of GPCRs via a G_q/IP_3 mechanism leads to rapid Ca^{2+} release from storage sites in the endoplasmic reticulum closely opposed to mitochondria, which is why targeting biosensors to the mitochondria has become the most sensitive means to measure Ca^{2+} responses to GPCR/IP_3 activation.

Comparison of Aequorin/Photina to Cameleons, Camgaroos, and Pericams

In general, because of their different properties, the two major families of photoproteins—aequorin/Photina and the cameleons, camgaroos, and pericams—are used for different purposes (see Table 1 for comparison of properties). Lower levels of emission of light of bioluminescent photoproteins compared to fluorescent proteins have for a long time restricted them to *in vitro* use. In addition, aequorin and Photina require coelenterazine as a cofactor, so they are primarily employed *in vitro*, since it is difficult to supply coelenterazine to target cells *in vivo*. Furthermore, both aequorin and Photina are one-time users: once Ca^{2+} binds to them they become inactivated and are exhausted as probes. As a consequence they cannot be usually employed to measure multiple Ca^{2+} signaling events in the same cell, especially if the first calcium wave consumes all the available aequorin. In contrast, the cameleons can be used with traditionally available fluorescence equipment and can regenerate once Ca^{2+} dissociates from the M13 and calmodulin binding sites. As a consequence, they can be used to measure continuous Ca^{2+} oscillations in a cell over time (with the limitation of photobleaching), and since they do not require cofactors can be used *in vivo* without the need to preload the tracer.

Furthermore, aequorin at most gives off one photon per molecule. And, as a consequence the bioluminescence is weak and very dim. This makes is difficult to image aequorin emission, and the protein in general provides poor spatial resolution. This limits its utility for basic cell biological research investigating Ca^{2+} transients in microdomains. In contrast, the high energy emission of the cameleons allows good spatial resolution, making them the probe of choice for basic research studies.

Both aequorin and Photina are bioluminescent probes and can be used in luminescent assay systems. Further, as mentioned above, the high energy emissions of Photina can be detected in both luminescent (such as CCD detectors) and fluorescent (such as FLIPR) devices. Both aequorin and Photina have low background signals, which is a highly advantageous property for HTS of drugs. In contrast, because camelons, camgaroos, and pericams are fluorescent emitters, they require light excitation, and as a consequence they have relatively high background signals compared to the bioluminescent emitters. Thus, the GFP sensors are not commonly used in HTS but instead are employed primarily in single-cell research efforts as well as *in vivo*, where they can be detected with two-photon detectors as well as other devices, which diminish the drawbacks of high background fluorescence.

Other properties of these two families of photoproteins also serve to primarily restrict their use for either drug screening or research. For example, the cameleons are relatively large molecules, consisting of two fused GFP molecules. Insertion of such large proteins in limited compartments could affect intracellular signaling processes. While they are generally believed to be nontoxic to cells, they can exert Ca^{2+} buffering, which can affect responsiveness of cells to GPCR stimulation. Evidence also exists that their sensitivity can be affected by intracellular pH,[77] which can be another factor limiting their use for drug screening. It is likely that some of these problems may be overcome in time with the development of newer cameleons so that these proteins may have greater application for drug discovery than existing probes.

Photoproteins as Tools to Measure Calcium Transients *In Vivo*

While aequorin and GFP Ca^{2+} sensors have been used extensively in isolated cells, recent data have led to the possibility of measuring Ca^{2+} signaling in intact living animals. The noninvasive measurement of Ca^{2+} transients is particularly important to study the relationship among neuronal activity, Ca^{2+} signaling, and behaviors. Furthermore, it provides a means to study neuronal or other cellular networks of multicellular signaling pathways, in a manner not readily assessed using other approaches. Ca^{2+} signaling in animals can also be employed in the future for drug discovery and as a biomarker for activity of drugs.

Photoproteins and GFP-Ca^{2+} sensors have been recombinantly expressed in whole animals using a variety of transgenic approaches. This has facilitated measurement of Ca^{2+} signaling in worms,[79,80] *Drosophila*,[81,82] zebrafish,[83] and mice.[84–86] In most of these studies, cameleons or camgaroos were expressed in the animals, and tissue slices were prepared and used for Ca^{2+} measurements. Two recent studies have also shown that photoproteins can be used to detect Ca^{2+} transients *in vivo* in the conscious mice.

Rogers *et al*.[86] generated transgenic mice expressing a GFP-aequorin fusion protein targeted to the mitochondria to measure intracellular Ca^{2+}. To generate a functional aequorin, they infused coelenterazine into the animal and measured Ca^{2+} transients as bioluminescent images using either the "Photon Imager" or the "Video Imager," consisting of intensified CCD cameras. Using this system, they measured corresponding increases in Ca^{2+} in muscle cells during sciatic nerve-stimulated contractions and detected oscillations that were related to both single twitches as well as the development of tetanus. Furthermore, whole-body scans of Ca^{2+} signaling in conscious freely moving mice, as well as in mice subjected to epileptic seizures, showed that changes in Ca^{2+} levels during the sleep–wake cycle could be detected. Interestingly, in many cases, synchronized wave patterning of Ca^{2+} oscillations were observed that were consistent with activation of networks of cells.

These studies are of some importance for both basic science purposes and drug discovery in that they could be employed to measure activity of specific cellular networks of cells while simultaneously measuring organ function and/or whole animal behavior. This would provide approaches that couple the function of specific cell populations to systemic physiology.

An obvious extension of the approach is that the technology could be used to test efficacy of GPCR compounds by screening their effectiveness to activate Ca^{2+} signaling *in vivo*. This would be especially useful in investigating the specificity of action of compounds by determining if they act at discrete sites *in vivo* coincident with tissues in which their target GPCRs are localized. A limitation of the approach highlighted by Rogers *et al*.[86] was that *in vivo* imaging of Ca^{2+} signaling in brain was not detected. This occurred despite the construct being expressed in brain. The authors suggested two reasons to explain this disparity: first, that coelenterazine does not easily access the brain following peripheral administration; and second, that the light emission from aequorin did not easily penetrate through deep organs or the skull.

These problems may be partly overcome by the use of the GFP Ca^{2+} sensors such as the camgaroos or pericams. Hasan *et al*.85 generated transgenic mice expressing these proteins under a *tet* promoter and used two-photon microscopy to detect Ca^{2+} signaling. These proteins abrogated the issues of using coelenterazine in an aequorin-based assay, and the use of two-photon microscopy alleviated some of the problems associated with signal penetration in deep tissues. With this approach they detected Ca^{2+} images *in vivo* in cortical tissue. However, at present this approach is primarily intended for basic research and would require extensive improvements to be employed for drug screening.

Conclusions

Because of the important role that Ca^{2+} plays in GPCR signal transduction, technologies that measure intracellular levels of this ion have served as the foundation for many GPCR drug discovery programs. While fluorescent dye probes and FLIPR devices have been used extensively for GPCR drug discovery over the last decade, this approach may be giving way to newer approaches to measure Ca^{2+}, particularly those that employ the photoproteins aequorin and Photina.[87,88] These photoproteins provide many advantages over the fluorescent dyes as probes for Ca^{2+}. First, they are cleaner. The background signals of aequorin and Photina are very low, and the dynamic range of measure is greater than dyes. Second, their increased strength of emissions mean fewer cells are needed for drug screening, and devices that facilitate automation of screening can be employed such as CCD cameras. Finally, their sensitivity is much greater than dyes, and this is due in part to the ability to target these photoproteins to discrete subcellular sites in cells, where changes in Ca^{2+} transients are much easier to detect in a defined and precise manner without the diffusion of the signal when Ca^{2+} changes are measured in the entire cytosol.

The photoproteins provide approaches to discover new types of drugs. By targeting the biosensors to distinct locations in cells, options become available to identify drugs that affect some functions of GPCRs but not oth-

ers. Furthermore, because of the improved sensitivity and limited background of these probes (aequorin/Photina), HTS for agonists, antagonists, and allosteric modulators becomes easier, as does screening for drugs targeting orphan GPCR. Finally, use of photoproteins opens up the possibility of measuring GPCR-modulated Ca^{2+} transients *in vivo* in animals. Not only will this be useful in target validation and in *in vivo* assessment of drug efficacy, it may provide the means to study GPCR signaling in discrete cell populations and networks *in vivo*. This will help both in understanding better the functions of GPCRs is selective tissues, but importantly may also provide unique approaches to discover designer drugs that are organ or cell population selective. Such novel opportunities hopefully will facilitate the imagination of researchers and expand their capabilities to discover and develop novel therapeutics to treat a host of diseases and disorders for which no effective therapeutics are presently available.

Acknowledgments

The authors wish to thank Dr. Vincent Dupriez, PerkinElmer Cellular Sciences Belgium SPRL, for his helpful comments on the manuscript.

Disclosure Statement

R.M.E. is an employee of PerkinElmer. No competing financial interests exist.

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Address reprint requests to:
Richard M. Eglen, Ph.D.
Bio-discovery
PerkinElmer Life and Analytical Sciences
940 Winter Street
Waltham, MA 02451

E-mail: richard.eglen@perkinelmer.com