Monitoring receptor signaling by intramolecular FRET

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A large variety of techniques has been used to monitor activation of G protein-coupled receptors (GPCRs) both in isolated membranes and in intact cells. However, most of these techniques cannot resolve receptor activation and signaling in space and in time. Here, we describe techniques that allow the temporally and spatially resolved monitoring of these processes by optical recording with energy transfer techniques. Fluorescence and bioluminescence resonance energy transfer, FRET and BRET, are based on energy transfer between two closely spaced probes. The exquisite sensitivity of FRET and BRET to the distance of the two probes makes these techniques ideal tools to study either protein–protein interactions (when the two probes are localized on two different proteins) or conformational changes within a given protein (when the two probes are localized on a single protein). Here, we review the latter approach as a tool to study receptor activation and the levels of the second messengers cAMP and cGMP in intact cells.

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Current Opinion in Pharmacology 2007, **7**:547–553

This review comes from a themed issue on
New technologies
Edited by Terry Kenakin and Paul Domanico

Available online 4th October 2007

1471-4892/$ – see front matter

DOI 10.1016/j.coph.2007.08.007

Introduction

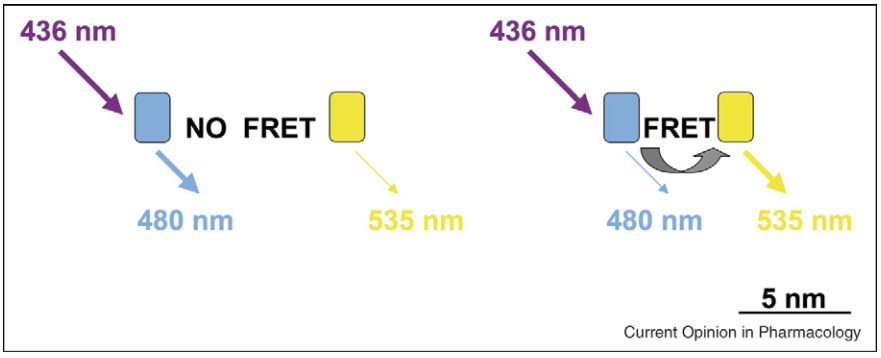
The pharmacology of G protein-coupled receptors (GPCRs), their activation and their biochemical and physiological effects, are probably the most studied topics in pharmacology, and a wealth of methods has been developed over the years to study these processes at many different levels, ranging from molecular mechanisms to overall physiological responses [1]. These methods can be, very roughly, subdivided into physiological and biochemical techniques. Physiological techniques use mostly intact organs or tissues and often permit repeated and long-term measurements, and they suffer and benefit at the same time from the fact that they often integrate a number of effects that occur downstream from receptor activation [2]. Biochemical techniques, on the other hand, are mostly used on disrupted samples (such as cell membranes) or require destruction of individual samples [3] and therefore generally contain little temporal and usually no spatial information. Thus, while we know a lot about the detailed mechanisms of receptor-mediated signaling on the one hand and about overall physiological responses on the other, we know very little about the generation and propagation of receptor signals in space and in time.

Several laboratories have, therefore, set out to develop methods that would provide just this: ways to monitor receptor activation and signaling in space and in time *in situ*, that is in intact cells and ultimately also in intact organs or organisms. These methods are based on optical techniques and all require fluorescent sensors that permit the visualization of such processes. These sensors consist of either the entire relevant protein(s) or of their critical parts – such as a cAMP binding domain – plus two fluorescent probes that serve as indicators of relative movement. In principle, any fluorescent probes can be used for this purpose, but in recent years the use of green fluorescent proteins (GFPs) and their color variants [4,5] has become the most important strategy, because this allows the entire sensors to be encoded and to be expressed in cells by simple DNA transfection. This technology was pioneered by Tsien and co-workers, who designed calcium sensors that they called cameleons. These sensors are based on calmodulin – a protein that changes its conformation upon binding of calcium – to which two fluorescent proteins were attached [6].

The principle of resonance energy transfer (RET) experiments

In the late 1940s, Theodor Förster discovered the transfer of energy from one dye to another one if the two are spaced closely together; closely means, in most instances, a distance below 10 nm. This transfer of energy, which is generally called Förster resonance energy transfer, occurs without radiation. If the two dyes are both fluorescent, it is called fluorescence resonance energy transfer, or short FRET [7]. The principle is depicted in Figure 1, using the now widely used GFP variants cyan (CFP) and yellow (YFP) as examples. In this case, excitation of CFP, which is best achieved with light of a wavelength of 436 nm, normally leads to emission of the typical cyan fluorescence, with a peak at a wavelength of 480 nm. If, however, a second fluorophore such as YFP is close by,

Figure 1



Principle of FRET using CFP and YFP as a donor–acceptor pair. FRET occurs when the donor (CFP) is close enough (<10 nm) to the acceptor (YFP), so that upon excitation of CFP (with light at a wavelength of 436 nm), energy is transferred to YFP and causes yellow emission (at 535 nm); at the same time, cyan emission (at 480 nm) decreases.

then – under appropriate circumstances – energy can be transferred to YFP and a yellow emission from YFP occurs that has a peak wavelength of 535 nm. In this transfer of energy, CFP acts as the donor and YFP as the acceptor. FRET depends on a number of parameters. In particular, it is exquisitely sensitive to the distance of the two fluorophores; the efficiency of FRET declines with the sixth power of the distance, with half maximal transfer occurring in many cases at a distance of about 5 nm. Therefore, FRET can serve as a 'molecular ruler' for distances in this range—which is about the size of many proteins [8].

In a variant of FRET, YFP is replaced by the small fluorescein derivative FlAsH, which binds to short cysteine-containing sequences and permits the use of a label much smaller than GFPs [9]. Another technique uses light-emitting luciferases as the donors instead of light excited CFP; this technique, which was pioneered by Bouvier and co-workers in the GPCR field mostly for studies of GPCR dimerization [10], has the advantage of reducing nonspecific signals that may be caused by excitation with light in the case of FRET, but has the disadvantage of lower emission intensities and, hence, lower spatial and temporal resolution [11,12].

It should be noted, though, that FRET depends not only on the distance of the two fluorophores, but also on their spectral overlap and their relative orientation, and that the readouts can be affected by a number of parameters such as fluorescence quenching which require a number of careful controls. These issues have been reviewed extensively (see, e.g., [13–16]) and are beyond the scope of the present manuscript.

Furthermore, even when the structures of the proteins to which the probes are attached are known, the structure of the overall sensor is usually not. Particularly in the case of GFP variants, the probes are large compared to the movements that they report, and they are quite flexibly attached to the proteins of interest, allowing for wide differences between the distances of the two probes and their attachment points. All this means that while FRET (or BRET) measurements indicate distances between the two probes, they do in general provide only relative information about distances between the attachment points in the protein(s) of interest. Therefore, these measurements, on their own without other structural information, are not really suited to elucidate molecular movements. However, they do indicate that movements happen, and they reliably report when and where in a cell they happen.

The two probes that allow FRET (or BRET) to occur can be placed either on two separate proteins or in two different locations of the same protein. The first strategy allows the monitoring of protein–protein interactions, since FRET will occur when the two proteins carrying the different probes associated and will be reduced or disappear when the two proteins dissociate. The latter strategy, in contrasts, records conformational changes that result in changes in either the distance or the orientation of the probes (and it is usually difficult to experimentally distinguish between these two potential mechanisms; see for example [17]). Even though the strategy of two probes attached to a single protein produces smaller changes in FRET than the first strategy (since the two probes will rarely move far away from each other to cause full loss of FRET), it has several advantages: The two probes are always expressed at a 1:1 stoichiometry, changes in the localization (for example translocation to the cell membrane) and resultant artifacts (such as fluorescence quenching) will affect both probes at the same time, and intramolecular FRET will usually outweigh more complex interactions. Therefore, this review concentrates on approaches that use sensors consisting of a single protein and two probes, that is monomolecular sensors, to monitor activation and signaling of GPCRs.

Analysis of receptor activation by FRET

When GPCRs bind agonists, they are thought to change into an active conformation, which in turn binds to and activates G proteins. The active conformation is also the form that is recognized and phosphorylated by G protein-coupled receptor kinases (GRKs), β-arrestins and the internalization machinery. While classical receptor activation models have assumed that there is just a single active (and a single inactive) conformation, more recent data suggests that several distinct conformations may exist, which may even show preferential interactions with specific downstream components (see below).

This conformational change into the (or a) active conformation has been studied by a variety of techniques. Earlier studies have used mostly biochemical, mutagenesis and spectroscopic methods [18–20]. All of these studies indicate that receptor activation leads to a relative rearrangement of the receptor's transmembrane helices, particularly of helix III and VI (reviewed by [21]). A special case in several respects is the 'light receptor' rhodopsin. Activation of rhodopsin by light leads to a sequence of conformational changes that starts with isomerization steps in its ligand retinal (which occur within femtoseconds) and ultimately leads to the active G protein signaling metarhodopsin II conformation; crystal structures have been analyzed for the inactive as well as several intermediate active conformations and allow a more detailed view of the activation steps and their conformational characteristics (reviewed by [22–24]). Studies with purified and fluorescently labeled β_2-adrenergic receptors have confirmed the concept of agonist-induced relative movements of helices III and VI and have further suggested the existence of intermediate states and sequential activation of receptors via these intermediate states [25–27].

On the basis of these observations, we have set out to generate FRET sensors of various GPCRs where fluorescent probes were inserted in the third intracellular loop and the C-terminus, respectively. This was done assuming that placement of a probe in the vicinity of the transmembrane helix VI that moves during receptor activation would lead to receptor sensors that respond to agonist activation with a change in FRET. The probes used were either CFP and YFP [28] or CFP plus labeling of specific, cysteine-containing sequences introduced into the third loop of receptors with the small, yellow emitting fluorescein analogue termed FlAsH (for fluorescein arsenical hairpin binder; [9]). Both of these techniques lead to similar results; labeling with FlAsH requires an extra step and the resultant CFP/FlAsH receptor sensor bleaches more rapidly, but it has the advantage of leaving the receptor more intact in terms of its ability to signal to G proteins and in several cases gave a sensor with a larger agonist induced change in FRET compared to the original CFP/YFP sensors [9,17,29]. Such FRET receptor sensors have been constructed for a number of receptors, including the α_{2A}-adrenergic, β_1-adrenergic, A_{2A}-adenosine, bradykinin and PTH-receptors [9,17,28,30,31].

So what can we learn from these receptor sensors?

First, all of the sensors reported to date respond to agonists with a decrease in FRET. This would be compatible with the notion that the third intracellular loop and the C-terminus of these receptors move apart during activation – as has been predicted from mutational and computer modeling studies of the α_1-adrenergic receptor [32]. However, given the caveats mentioned above about the structure of such sensors, this may in fact be more a chance co-incidence and the FRET studies should not be used to make predictions or draw conclusions about the molecular mechanisms of receptor activation.

Second, all receptor sensors have revealed interesting kinetic data. While it is at present impossible to say with certainty whether the attachment of fluorescent probes affects the kinetics of conformational changes in these receptors, a number of indirect data show that at least some of these receptor sensors still retain roughly normal signaling properties and that, in these instances, downstream signaling events are triggered with the expected kinetics (for example [9,28]). For example, it has been shown that an α_{2A}-adrenergic receptor sensor labeled with CFP and YFP triggered opening of GIRK potassium channels with similar kinetics as did the corresponding native receptors. Thus, the kinetics of these receptor sensors should reflect, at least with some approximation, the kinetics of the native receptors.

The kinetics of the changes in FRET of such receptor sensors in response to agonists have always been quite rapid and have followed a mono-exponential time course. In most cases they occurred with a rate constant τ of 30–50 ms—for example in the A_{2A}- adenosine, the α_{2A}- and β_1-adrenergic receptors [9,17,28,31]. These rate constants are faster than had been assumed earlier—but on the other hand, they are considerably slower than those measured for rhodopsin, where formation of the metarhodopsin II form takes about 1 ms [23]. The only exception that we have encountered so far is the PTH receptor, where activation occurred with a time constant of ≈1s [28]; this may, however, be related to the complex and slow binding of the large ligand, PTH [33]. Slow activation kinetics have also been described for a similar bradykinin receptor sensor [30], but the different setup used by these authors makes a direct comparison difficult. Our own experience shows that optimal placement of the superfusion system is absolutely essential to obtain reproducible data on the speed of receptor activation.

Third, there is some evidence to suggest that the speed of the conformational change that is seen from the FRET

signal may depend on the type of ligand used. Studies with the α_{2A}-adrenergic receptors showed remarkably different speeds for inverse agonists, partial and full agonists [17,29]. The conformational changes induced by partial agonists, which produce signals of only partial amplitude, were considerably slower than those induced by full agonists [29], and inverse agonist signals, which resulted in an *increase* in FRET (versus a *decrease* caused by full and partial agonists) were even slower [17]. The time constants varied by a factor of about 25—the half-times of activation being ≈1s for inverse agonists and very partial agonists, but only ≈40 ms for full agonists. These observations are in line with the hypothesis that the different compounds may induce distinct conformations of the receptors, and that the switching times from the resting state may be distinct for all of these conformations. This hypothesis is in agreement with the studies on purified, chemically labeled receptors by Kobilka and co-workers [25–27]. They further agree with the emerging topic of 'biased agonism' that refers to ligands having differential efficacy in various downstream effects [34,35]. However, our data do not allow conclusions as to whether the full agonists switch the receptors rapidly through a set of partially active conformations (as suggested by the studies with isolated receptors) or whether the distinct active conformations might be achieved directly via distinct switching modes.

Very little work has been done with respect to spatial resolution of receptor activation in intact cells or tissues, even though ratiometric FRET imaging as well as fluorescence lifetime imaging methods can very well resolve such signals at the microscopic level. Future studies will have to show the potential of such approaches.

Analysis of cAMP signals by FRET

Signals downstream from the activation of receptors have also been recorded by appropriate FRET sensors, but all of these assays involve sensors with probes on different proteins (such as receptors and G protein subunits, two different G protein subunits—see for example [36–41]) and will not be reviewed here.

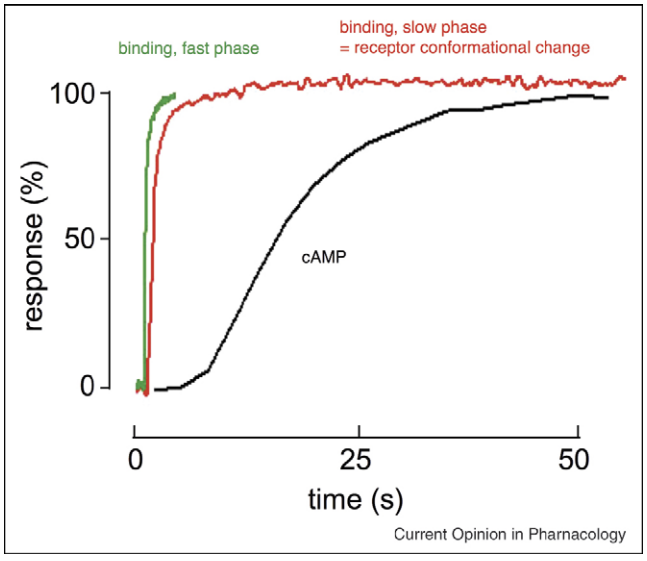
An aspect of GPCR signaling that has been studied quite extensively with monomolecular sensors is the monitoring of intracellular cAMP levels (reviewed by [42]). Optical recording of intracellular levels of cAMP was first achieved with multi-protein sensors based on protein kinase A (PKA), a protein that dissociates in response to binding of cAMP into a dimer of regulatory (R) subunits and two catalytic (C) subunits. Chemical labeling of the purified subunits with fluorescein (Fl) and rhodamine (Rh) resulted in a sensor termed FlCRhR (pronounced 'flicker') that could be injected into intact cells to monitor their cAMP levels. Upon binding of cAMP, the FlCRhR sensor dissociated resulting in a loss of FRET between fluorescein and rhodamine [43]. While this sensor has been used successfully in several cell types including intact neuronal networks, the need to biochemically prepare and to microinject the sensor precluded its widespread use. Therefore, the generation of similar sensors using GFPs instead of fluorescein and rhodamine was a significant improvement [44]. These sensors have allowed the detailed study of intracellular cAMP kinetics and have, in particular, been used to study localized changes of cAMP in cardiac myocytes (see below). However, they suffer from the disadvantages of multi-protein sensors mentioned above (i.e. they require stoichiometric expression of two labeled proteins; the two proteins may become localized differently). In addition, they may be further affected by the intact enzymatic activity of the catalytic subunit and are, finally, complicated by the fact that PKA dissociation and re-association are complex cooperative processes, which results in slower kinetics of the sensor compared to real cAMP kinetics.

As a consequence, several groups have attempted the generation of monomolecular cAMP sensors and have been successful using the cAMP effector proteins Epac (exchange proteins activated by cAMP; [45]). Epac consists of a regulatory cAMP-binding site and a catalytic domain, which serves as a GTP/GDP exchange factor for the small GTP-binding protein Rap1. Two groups independently created similar sensors, comprised either the entire Epac1 or a slightly truncated (lacking the membrane interacting domain) and catalytically inactive mutant, both sandwiched between CFP and YFP [46,47]. Binding of cAMP to these sensors induces a conformational change, which decreases FRET between the CFP and YFP. These sensors are faster, but also of lower affinity than the PKA-based multi-protein sensors [47].

Even simpler sensors, more like the cameleon Ca sensors mentioned in the introduction, can be generated by using just the cAMP-binding domains from either Epac-proteins, the regulatory domain of PKA or cAMP-activated ion channels, sandwiched again between CFP and YFP [48,49]. These sensors, termed camps (for cAMP sensors) have – like native PKA – cAMP affinities around 1 μM, react rapidly, are uniformly distributed in the cytosol of transfected cells and, because of their lack of catalytic and protein binding activities, are presumably relatively inert for the cells.

Whereas the receptor sensors discussed above have so far mostly been used to yield temporally resolved data, the most interesting results obtained with the different cAMP sensors concerns the spatial resolution of receptor signaling. In terms of temporal resolution, the cAMP sensors have, not surprisingly, shown that cAMP responses to receptor stimulation are relatively slow and occur with significant lag times (see Figure 2). Intracellular cAMP levels appear to be often regulated essentially by the degradation via phosphodiesterases [50,51], which are

Figure 2



Kinetics of parathyroid hormone (PTH) binding to its receptor, receptor activation and cAMP generation as derived from FRET experiments. Shown are the FRET signals resulting from labeled PTH (1–34) and labeled receptor ('binding'), double labeled receptor ('activation'), and epac1-sensor ('cAMP'). PTH(1–34), which is the active 34 amino acid N-terminus of PTH, shows biphasic binding to its receptor; the second, slower phase has the same kinetics as the conformational change as detected by intramolecular FRET of a receptor sensor. Data are from ref. [33].

expressed in a cell type-specific manner. And an interesting, as yet ill understood, phenomenon is the observation that intracellular Ca^{2+} and cAMP levels may show interdependent oscillations [52–54]. However, these oscillations have been observed to be inverse in insulin-secreting MIN6 cells [53], whereas they were parallel in transfected HEK293 cells [54], presumably reflecting a dominance of Ca-dependent phosphodiesterase activity in the first, and of Ca-dependent adenylyl cyclase activity in the second case.

The spatial resolution that can be achieved with cAMP sensors has been mostly used to study the long debated question on whether and how receptor-mediated signaling may be compartmentalized. Compartmentalization of cAMP has been postulated for many years in many cell types, most importantly perhaps in cardiac myocytes (see [55,56], for an overview). In these cells, there were a number of hints suggesting that receptor-mediated cAMP signals may be localized within a cell, and that there may be cAMP compartments that respond differently to different stimuli.

The genetically encoded PKA-based indicators yielded, for the first time, the visualization of microdomains with high cAMP-concentrations during adrenergic stimulation of neonatal cardiac myocytes, which were localized to the striated pattern of these cells at the site of PKA targeting by A-kinase anchoring proteins [57]. The formation of these microdomains was attributed to the high activity of different isoforms of PDEs present in cardiac tissue, mostly PDE3 and PDE4 [50,57]. Locally increased camp responses to adrenergic stimulation at the *Z*-lines were also reported in PDE4-knockout mice [58]. A potential problem of localized cAMP signals obtained with the PKA-sensor is the fact that the sensor itself is localized by binding to A-kinase anchoring proteins, which may affect the measurements.

Distinct cAMP compartments have, however, also been obtained with monomolecular cAMP sensors. DiPilato *et al.* [46] reported different kinetics of submembrane and nuclear cAMP levels with a differentially targeted, transfected Epac-based sensor [46]. Studies in cardiac myocytes with a camps sensor on the basis of the binding domain of a cyclic nucleotide channel expressed in the hearts of transgenic mice suggested that cAMP responses to β_2-adrenergic stimuli were localized to the site of stimulation, whereas those to β_1-adrenergic receptor stimulation propagated throughout the cells [49]. These studies indicate that there may be a spatial texture in signaling via GPCRs and cAMP, which we are just beginning to unravel.

On the basis of principle of the monomolecular cAMP sensors described above, but also using the entire or large parts of the cGMP-dependent protein kinase protein, it has been possible to create also sensors for cGMP, the second messenger generated by membrane bound as well as soluble guanylyl cyclases, that have different affinities and selectivities [59–61]. This opens up the possibility of studying not only a second messenger system, but perhaps – if differently colored probes can be attached – of monitoring two cyclic nucleotide second messengers at the same time.

An intramolecular β-arrestin sensor

A third type monomolecular sensor for GPCR signaling has been developed using the conformational change that occurs in β-arrestins when they get activated and bind to receptors. This conformational change appears to be a prerequisite for high affinity interactions with agonist-activated, GRK-phosphorylated receptors, which can then trigger both receptor internalization and non-conventional signaling such as activation of the MAP kinase cascade, and it presumably involves release of the C-terminal tail of β-arrestins [62]. To probe these changes in living cells, Bouvier and co-workers constructed an intramolecular BRET-based biosensor, in which β-arrestin2 is sandwiched between Renilla luciferase and YFP [63]. Intramolecular BRET between Luc and YFP was significantly increased in response to GPCR activation, in agreement with the proposed conformational rearrangement that would bring the N-terminus and the C-termi-

nus of β-arrestin2 in closer proximity. Kinetic analyses of these changes, which take place over minutes, were in very good agreement with the time courses of β-arrestin translocation from the cytosol to the cell membrane as revealed by translocation assays seen either by confocal microscopy or by FRET between β-arrestins and receptors [28,64,65]. Thus, this sensor could become a unique tool to study both the kinetic and the spatial patterns of non-conventional signals of GPCRs as well as the triggers of their internalization and desensitization.

Future perspectives

The possibilities of recording receptor signals in real time with high spatial resolution permit a new perspective on receptor function at various levels. A number of old – and new – questions can now be addressed from a new angle. A major topic will cover the relationships between the conformational change and its kinetics with specific downstream signaling events. A second field of research will concern attempts to localize receptor signaling—for example to look at specific sites of synaptic activity, or to look at compartmentalization of receptor activation and second messenger production. A third venue will be the simultaneous analysis of either two or more steps in a signaling chain, or of several second messengers at the same time, and a final point will be the transfer of these techniques into more physiological settings, including ultimately true *in vivo* imaging. All these aspects appear to be realistic with the techniques available today and will certainly reveal complexities in receptor signaling that we have not even been able to imagine in the past.

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