Review

Cell PRESS

Optical techniques to analyze real-time activation and signaling of G-protein-coupled receptors

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The activation of G-protein-coupled receptors (GPCRs) is traditionally measured either by monitoring downstream physiological events or by membrane-based biochemical assays. Neither of these approaches permits detailed kinetic or spatial analysis of receptor activation and signaling. Recently, several optical techniques have been developed to monitor receptor activation either by using purified reconstituted GPCRs or by observing GPCRs, G proteins and second messengers in intact cells. These techniques are providing, literally, new views on both the mechanistic basis of the signaling process and the kinetic and spatial properties of GPCR-mediated signals. They suggest that agonists can activate GPCRs within milliseconds, that different compounds can induce distinct active conformations of GPCRs, that G-protein activation is the rate-limiting step in GPCR signaling, and that cellular signals can be temporally and spatially confined. They are also raising controversial issues, such as whether or not receptors and G proteins are pre-coupled and whether G proteins dissociate during activation.

Introduction

Many fundamental issues concerning G-protein-coupled receptor (GPCR) function have remained unresolved despite decades of intensive research. Open questions cover simple and fundamental points. Where and when do GPCRs become activated? Do they switch in an 'on–off mode', or do they have multiple active conformations? How rapidly can they be switched? Are their signals spatially and temporally encoded? Do GPCRs function as monomers or oligomers, are they pre-coupled to G proteins and/or do they form higher ordered complexes?

Until recently, the optical techniques needed to answer some of these questions have been available only for the 'light receptor' rhodopsin, owing to its abundant expression in the retina and changes in the photo-properties of its covalently bound ligand, retinal, during activation. Spectroscopic investigations of rhodopsin have revealed that light induces a series of conformational changes that result in distinct active conformations until – within a millisecond – the fully active, G-protein-stimulating form, metarhodopsin II, is attained [1]. Various spectroscopic assays have been developed for subsequent signaling steps of the rhodopsin system; these assays show rapid transduction of the signal through G proteins to the cGMP phosphodiesterase, leading ultimately to a closure of cGMP-gated channels – all within 100–200 ms [1]. It is not clear, however, whether rhodopsin represents a special case owing to its highly specialized function or whether its mode of activation is prototypical for GPCRs.

Recently, several optical techniques, mostly based on fluorescence, have been developed for other GPCRs and have begun to shed light on the activation mechanisms and the temporal and spatial patterns of GPCR signaling.

Techniques for labeling GPCRs

Unless receptors are purified, all optical studies require the introduction of labels. The most popular labels are either fluorescent or luminescent. Although small fluorescent labels can be introduced into membrane proteins expressed on the surface of intact cells [2], we are not aware of attempts to use this approach for GPCRs. Only purified β_2-adrenoceptors have been chemically labeled with fluorescent ligands and subsequently reconstituted into phospholipid vesicles [3].

For studies in intact cells, GPCRs have been mostly fused with green fluorescent protein (GFP) and its color variants, most importantly the cyan (CFP) and yellow (YFP) fluorescent proteins [4]. GPCRs labeled with GFP at the carboxyl (C) terminus were used in initial studies to investigate by fluorescence microscopy the membrane targeting and intracellular trafficking of these receptors [5]. Subsequently, they have been used to assess dimerization by attaching two different GFP variants to the same or two different types of receptor and investigating their interactions [6,7]. More recently, two different GFP variants have been inserted into receptors and/or G proteins to study activation kinetics and to image spatial patterns of GPCR activation and signaling [8,9]. In all of the latter studies, the measurements were based on fluorescence resonance energy transfer (FRET) – a technique that

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0165-6147/$ – see front matter doi:10.1016/j.tips.2007.12.002 Available online 11 February 2008

Box 1. FRET in GPCR research

Fluorescence resonance energy transfer (FRET) occurs between a 'donor' and an 'acceptor' label; these labels can be attached to a single protein or to two different proteins. FRET requires spectral overlap (i.e. the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor), a close distance (<10 nm) and an appropriate orientation (which is usually unknown and assumed to be random).

In the most popular design, FRET occurs between cyan (CFP) and yellow (YFP) fluorescent proteins. For example, these labels can be attached to a single GPCR (in the third intracellular loop and the C terminus; Figure I, left) or to a GPCR and one of the subunits of a G protein (right). Excitation of CFP with light at 436 nm causes CFP emission at 480 nm plus FRET to YFP, which then emits at 535 nm. The extent of FRET varies with the sixth power of the distance and is thus an exquisitely sensitive indicator of conformational changes (left) or protein–protein interactions (right).

Addition of an agonist (red triangle) presumably changes the distances between CFP and YFP; it can induce a rapid reduction in FRET in a single receptor labeled with CFP and YFP (left; CFP emission increases and YFP emission decreases), and can promote the interaction between a YFP-labeled receptor and a G protein labeled at its γ subunit with CFP (CFP emission decreases, YFP emission increases).

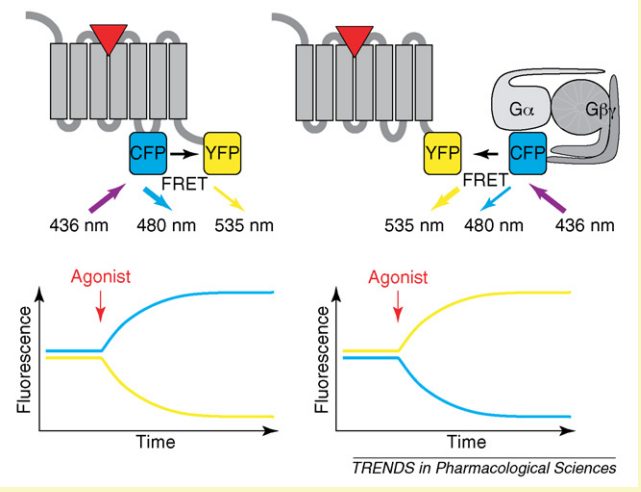

Figure I. Principles of FRET in a single receptor (left) and between a receptor and a G protein (right).

can be used to determine small changes in distances [10] when the labels are ~5 nm apart (Box 1).

A variant of GPCR labeling uses a light-emitting enzyme, luciferase, as a donor and a GFP variant as an acceptor in bioluminescence resonance energy transfer (BRET) studies [11]. Because the use of luciferase avoids the need for light illumination, BRET is often characterized by low background; however, the low intensity of emitted light essentially precludes imaging or the monitoring of very rapid kinetics.

A recently developed labeling alternative is to fuse an enzyme, either O^6-alkylguanine-DNA alkyltransferase (AGT) [12] or the acyl carrier protein (ACP) [13] to a protein of interest. The enzyme can then be labeled by activated small organic dyes by means of its enzymatic activity. AGT labeling has been applied to track neuropeptide-Y receptors [14], whereas the ACP tag has been used in FRET microscopy to demonstrate the monomeric nature of neurokinin-1 receptors [15].

All of these labels are relatively large (up to 27 kDa); as a result, attempts have been made to find smaller fluorophores for labeling proteins in intact cells. The fluorescein arsenical hairpin binder (FlAsH) and its red variant (ReAsH) are small fluorophores that can specifically label tetracysteine motifs in intracellular proteins. These small dyes can penetrate cell membranes and bind to proteins containing the core binding sequence CCPGCC or optimized variants [16]. FlAsH has been used to label correspondingly mutated GPCRs [17] and can 'replace' the much larger YFP molecule in FRET studies with CFP [17–20]. Because the labeling and washing procedure is difficult, however, this technology has been used by only a few laboratories.

Optical analyses of GPCR activation and signaling

One of the main advantages of optical readouts as compared with biochemical parameters of GPCR signaling is the potential for continuous, real-time monitoring. This possibility has led to several studies that have resolved GPCR activation and signaling in time and in space to a much greater extent than was previously possible. In this section, we address the key results and controversial issues.

Kinetics and limiting steps of GPCR signaling

Labeled GPCRs are uniquely suited to follow many steps of the GPCR signaling chain in real time in intact cells. Such studies have led to a re-appraisal of the kinetics of GPCR signaling. Most importantly, the kinetics is significantly faster in intact cells than in isolated membranes or reconstituted systems [8]. Fluorescent or luminescent labeling of ligands, receptors and G-protein subunits, coupled with the development of FRET and BRET sensors for second messengers, has allowed the determination of kinetic parameters for many steps of the signaling cascade (Figure 1), including ligand binding [21,22], receptor activation [17,23,24], receptor–G-protein interaction [18,19,25,26], G-protein activation [26–28], effector activation [28] and cAMP concentration [29–31]. The fast kinetics of the initial steps of the signaling chain allows reconciliation with the rapid control of ion channels by G proteins [28,32]. FRET and BRET assays also permit determination of the speed of β-arrestin binding, and thus desensitization and triggering of non-classical signaling pathways; here, the rate limiting step is GRK-dependent phosphorylation (reaction half time, $t_{1/2} \approx 20$ s in HEK293 and A431 cells [33,34]), which is followed by rapid binding of β-arrestin-1 or β-arrestin-2 ($t_{1/2} \approx 2$ s) [34,35] and a subsequent slow conformational change in β-arrestin ($t_{1/2} \approx 5$ min under conditions where β-arrestin binding to receptors had $t_{1/2} \approx 50$ s) [36].

Unfortunately, there is no single GPCR system for which the whole signaling cascade has been monitored. The kinetic parameters shown in Figure 1 are derived from FRET and BRET experiments using several class-A GPCRs, such as the α_2- and β_1-adrenoceptors and the A_{2A}-adenosine receptor, with small agonists. The kinetics of agonist binding has so far been measured only for two receptors that bind larger ligands – namely, the neurokinin-2 (NK2) receptor [21] and the parathyroid hormone

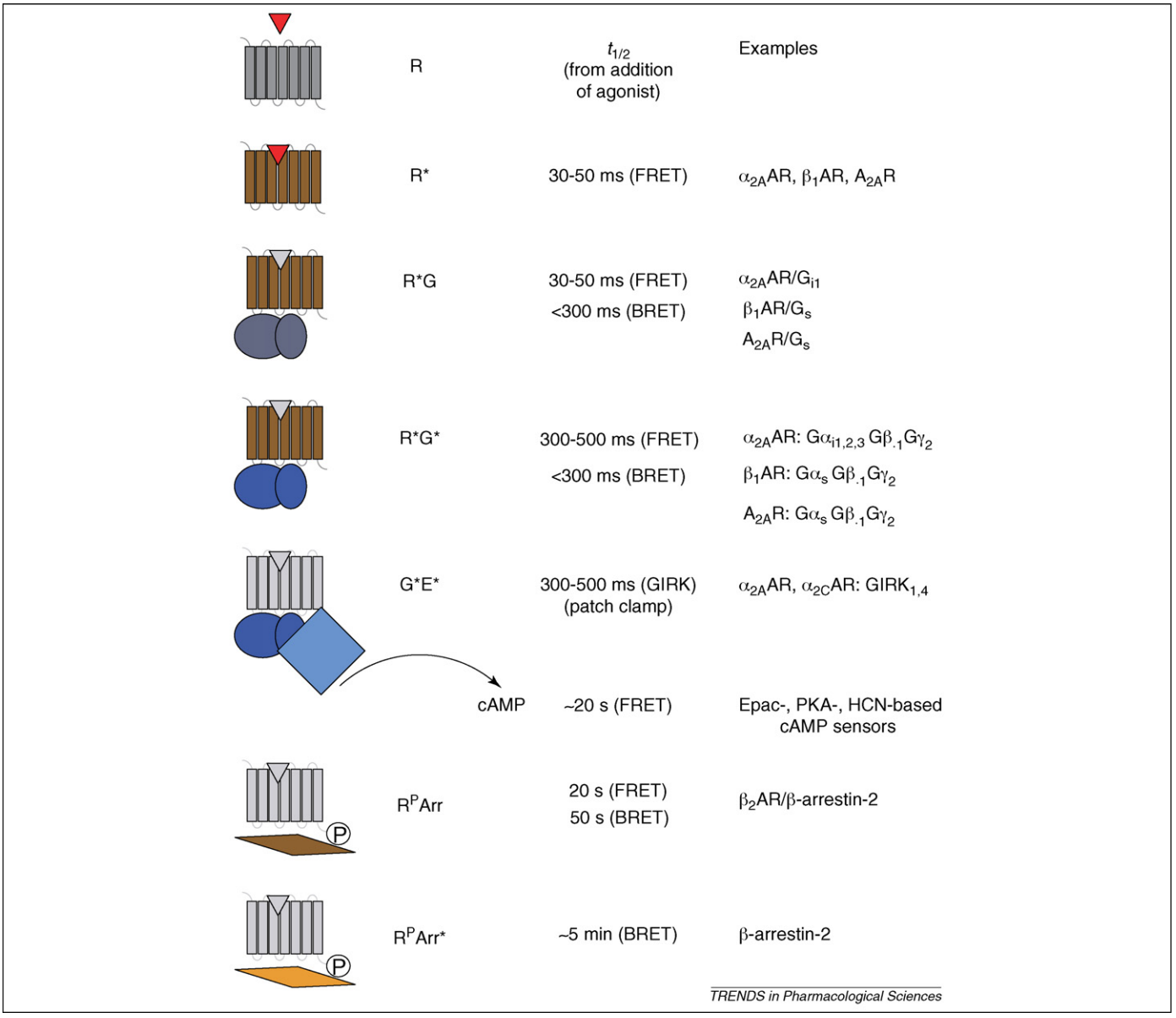

Figure 1. Kinetics of the steps in GPCR-mediated signaling. Stimulation of a GPCR (R) with agonist (red triangle) leads to an active receptor conformation (R*); interaction of the receptor with a G protein (G) and its activation (G*); activation of an effector (E*) by the active G protein; subsequent binding of β-arrestin (Arr) to the GRK-phosphorylated receptor (R^P); and lastly, a conformational change in β-arrestin (Arr*). The active components in each step are colored. The $t_{1/2}$ values are derived mostly from FRET and BRET experiments in intact cells and denote the times from the addition of an agonist (at saturating concentrations) to the respective step. All values refer to situations where agonist concentrations and expression of all signaling components are high. Data are compiled from Refs [17–20,23–31,34–39]. Abbreviation: HCN, hyperpolarization-activated cyclic-nucleotide-gated channel.

(PTH) receptor [22]. For both receptors, agonist binding was found to be biphasic and slower than the receptor activation kinetics shown in Figure 1. The faster phase was strictly dependent on agonist concentration ($t_{1/2} \approx 100$ ms for PTH and $t_{1/2} \approx 300$ ms for neurokinin A at maximal concentrations). It was followed by a slower phase with saturable speed ($t_{1/2} \approx 1$ s for PTH and $t_{1/2} \approx 14$ s for NK2 receptors). For the NK2 receptor, it was suggested that the two rate constants reflect two distinct conformations of the receptor: the first one linked to Ca^{2+} signaling and the second to cAMP signaling [21]. For the PTH receptor, the fast phase was interpreted as a first binding step, presumably between the amino (N) terminus of the receptor and the C terminus of the ligand. The slower phase coincided with the PTH-induced conformational change deduced from intramolecular FRET in the receptor (Box 1) and might reflect interactions of the N terminus of the ligand with the transmembrane core of the receptor [22].

It is intriguing to note that, among the steps following ligand binding (Figure 1), the receptor conformational change and the subsequent receptor–G-protein interaction occur with similar speeds (provided that enough receptors and G proteins are present), whereas the next step – activation of G proteins – is approximately 10 times slower. G-protein–effector coupling can be deduced from studies that simultaneously monitor activation of G_i proteins by FRET and activation of Gβγ inwardly-rectifying K^+ (GIRK) channels by whole-cell patch-clamp recording [28]; these studies have shown that there is a close temporal correlation between G_i activation and GIRK channel opening. In

apparent contrast, in G_s-mediated stimulation of adenylyl cyclase, cAMP accumulation becomes visible only after approximately 2 s. Because direct assays of G_s and adenylyl cyclase interaction are lacking, however, it is not clear whether this observation is due to a slow protein–protein interaction or to a slow accumulation of cAMP. The speed of the latter process seems to be largely determined by the speed of cAMP degradation by phosphodiesterases [37], and the achievement of equilibrium might take a minute or longer.

Thus, the signaling chain contains steps that show the potential for kinetic regulation (agonist binding, G-protein activation and second messenger accumulation), whereas other steps seem to be almost 'instantaneous'. It should be noted that the kinetics measured depends on the levels of the components and that maximal rates are achieved only at high concentrations, as might occur physiologically at postsynaptic sites and experimentally in transfected cells.

Multiple active states of GPCRs

The question of whether there are multiple active states of GPCRs has been the subject of a whole issue of this journal (e.g. see Ref. [3]). A large contribution to this subject has come from elegant studies on isolated, fluorescently labeled receptors [3]. These studies suggest that, like rhodopsin, β_2-adrenoceptors are activated through a series of conformational states that differ in their ability to link to G proteins, G-protein-coupled receptor kinases (GRKs) and β-arrestins, and that are characterized by specific fluorescence changes at different sites of the receptors. Interestingly, partial agonists seem to trigger only the first steps, whereas full agonists drive the receptors to a fully active conformation capable of interacting with all of its downstream protein partners.

Kinetic FRET studies have extended this concept by showing that there is a relationship between the intrinsic efficacy of compounds and the kinetics of the conformational change in receptors or in G proteins. Studies with labeled α_2-adrenoceptors have revealed rapid conformational changes and G-protein activation for full agonists ($t_{1/2} \approx 50$ ms), progressively slower changes for partial agonists ($t_{1/2} < 1$ s), and slow changes ($t_{1/2} \approx 1$ s) in the opposite direction with inverse agonists [20,38]. Again, these differences might be interpreted as evidence for the attainment of distinct active states of the receptors; such states might also differ with respect to distinct downstream pathways; that is, a full agonist in one pathway might be a partial or even inverse agonist in another. As a result, both the amplitudes and the kinetics of changes in FRET can be used to classify compounds as full, partial or inverse agonists at GPCRs [20,38,39].

Protein–protein interactions in GPCR signaling

Receptor–G-protein pre-coupling. In a complex signaling chain involving multiple proteins and their subunits, it seems logical that the relevant proteins are in a close assembly, often involving scaffolding proteins that can hold large protein assemblies together. A key question thus concerns how and to what extent these 'receptosomes' are pre-assembled.

In this context, pre-coupling between GPCRs and G proteins is the most interesting and controversial issue. A collision-coupling model in which one receptor activates many G proteins has been deduced from the rhodopsin–G_t system, for which recent studies confirming classical experiments have given rates of ~600 G_t molecules activated per rhodopsin molecule per second at 22 °C, and ~1300 at 34 °C [40]. In the late 1970s, studies by Tolkovsky and Levitzki [41,42] established a collision-coupling model for the β-adrenoceptor system in turkey erythrocyte membranes, but found that adenosine receptors in the same system seem to be more tightly coupled. Such tighter coupling has been found for G_i-coupled receptors such as the D_2-dopamine receptor [43], but not the G_i-coupled A_1-adenosine receptor [44].

Recent BRET and FRET studies have yielded conflicting results. For the α_2-adrenoceptor–G_i complex, both a collision-coupling [18] and a pre-coupled [26,45] model have been proposed. FRET and BRET studies have proposed pre-coupling for several types of receptor [25,26,46,47], whereas collision coupling is compatible with others [48]. Pre-coupled models clearly contradict classical findings of signal amplification and spare receptors, which have been observed not only for rhodopsin [1,40] but also for the α_2-adrenoceptor [32]. These discrepancies call for further experiments directed at the different amplification steps. In might be that pre-coupling between receptors and G proteins occurs for some receptors, whereas free collision is used to couple others, and that these two possibilities simply represent extremes of the affinities that GPCRs have for G proteins.

It should be noted that both FRET and BRET measurements monitor spatial proximity rather than functional interaction. Thus, an agonist-independent signal indicates pre-assembly of the proteins of interest (i.e. receptors and G proteins), but this pre-assembly does not necessarily mean that the proteins are functionally coupled. Furthermore, a basal signal does not necessarily imply that a receptor–G-protein assembly is stable, because a rapid turnover is also compatible with a basal signal, provided that some receptors and G proteins are close enough for FRET (or BRET) to occur at any point in time. These considerations might help to resolve the apparently contradictory observations on the coupling between receptors and G proteins.

Receptor dimmers. A point that can be mentioned only briefly here, and which has been the subject of several recent reviews [6,7,49], is the question whether GPCRs function as monomers, dimers or even higher order oligomers. BRET and FRET experiments have been crucial in postulating dimer formation, because they have shown that the coexpression of many appropriately labeled GPCRs causes energy transfer between the labels, indicating close proximity. Because little or no changes are induced by agonists in many studies, dimer formation has been considered to be constitutive. For the α_{1b}-adrenoceptor, FRET studies with receptors fused to three different labels have led to the conclusion that this receptor might function as a higher order oligomer [50].

Although the correctness of such BRET experiments has been challenged [51], the general consensus is that these experiments do support dimer formation [52,53], which is, however, only one requirement that should be met before postulating a relevant GPCR dimer [7]. Thus, although it is clear that some GPCRs such as the γ-aminobutyric acid B ($GABA_B$) receptors function as (obligatory) heterodimers and the metabotropic glutamate receptors function as homodimers [54], recent results in highly characterized reconstituted systems show that both rhodopsin [55] and the β_2-adrenoceptor [56] can efficiently signal to G proteins as monomers. Again, it seems that the monomeric function and the tight dimeric or oligomeric function of GPCRs might simply be extremes of a spectrum of different affinities that GPCRs show for each other.

G-protein subunit dissociation versus re-arrangement. A third controversial point in GPCR signaling that results, among others, from FRET studies is the question of whether G proteins dissociate during their activation. On the basis of numerous studies using mostly cell membranes, it has been commonly assumed [57] that, when receptors trigger GDP–GTP exchange in the Gα subunit, the subunits dissociate into a GTP-bound Gα protein and a Gβγ complex, both of which can signal to downstream effectors [58]. Dissociation has also been suggested by the observation that different Gα subunits can share a pool of Gβγ [59].

This view has been challenged most clearly by experiments using a covalently tethered G protein in yeast that is still capable of signaling; these experiments indicate that GPCR–G-protein signaling does not necessarily require G-protein dissociation [60]. Similarly, FRET assays suggest that G_i-like G proteins might not need to dissociate to signal [28,61]. Although insertion of the label into the Gα subunit has been assumed to be responsible for this nondissociating behavior [62], other FRET and BRET studies in intact cells have led to the conclusion that at least some G proteins can signal in response to receptors or other stimuli without the need to dissociate [26,63–65]. Experiments with isolated G proteins further suggest that G proteins do not dissociate within the time frame relevant for signaling [66]. Although it might be too early to challenge the prevailing concept of G-protein dissociation, these studies call for renewed investigations of this topic.

Compartmentalization of GPCR signaling

Studies on second messengers often seem to imply that receptor-induced changes in their concentration are uniform across a whole cell. It is obvious, however, that temporal and spatial changes in second messengers would permit a much more elaborate regulation of cellular functions. Oscillations in free Ca^{2+} have long been established, and recently it has been shown with cAMP FRET sensors that changes in Ca^{2+} are linked to oscillations in cellular cAMP mediated by Ca^{2+}-dependent phosphodiesterases [67] or adenylyl cyclases [68]. The role of these oscillations in intact settings needs to be studied. A recent investigation using cAMP and protein kinase A (PKA) indicators has revealed that spontaneous oscillations occur in PKA

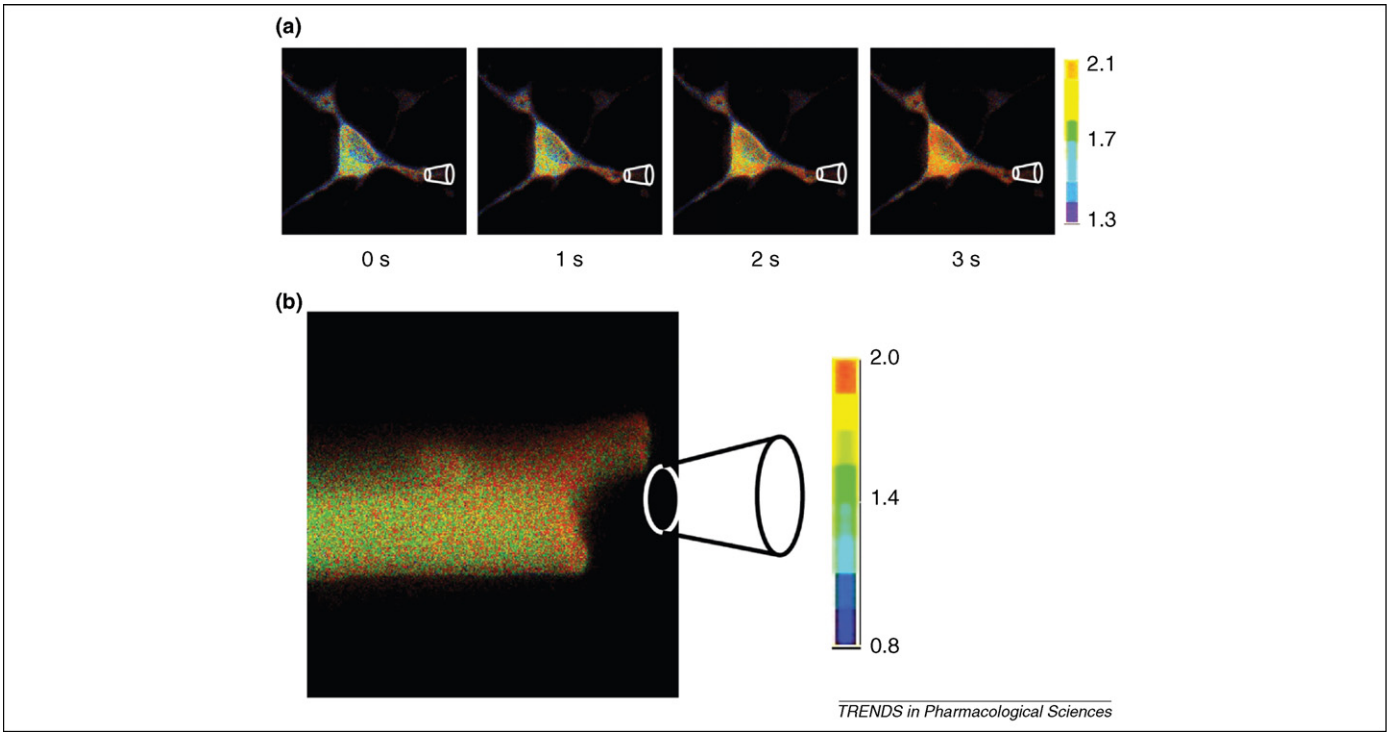

Figure 2. FRET imaging of localized versus generalized cAMP signals in neurons and cardiac cells. **(a)** Hippocampal neuron. **(b)** Cardiomyocyte. The FRET sensor for cAMP was expressed by transfection of isolated neurons (a) or by generation of a transgenic mouse (b). In both examples, production of cAMP was triggered by stimulation of endogenous β_2-adrenoceptors with a submaximal concentration of isoprenaline (100 nM; in [b], 100 nM CGP20712A was also added to block β_1-adrenoceptors), delivered locally through a patch pipette (shown schematically). Ratiometric images (YFP/CFP; see false color scale) were taken at the indicated times after agonist application (a) or after 3 s (b). It can be seen that the cAMP signal in the neuron is generalized; that is, it propagates throughout the cell after a localized stimulus to the β_2-adrenoceptors. In sharp contrast, cAMP signals generated by localized stimulation of the β_2-adrenoceptors in cardiomyocytes remain confined to the region of stimulation.

activity in neonatal rat retinal explants, and that these oscillations are temporally correlated with spontaneous depolarizations [69].

Spatial analysis of cAMP has been made possible, for the first time, by the injection of a PKA-based FRET sensor for cAMP into cells of simple neuronal circuits in both *Aplysia* sensory neurons [70] and the lobster stomatogastric ganglion [71], where patterns of cAMP transients were observed in response to electrical stimulation. In neuronal cells, diffusion of cAMP from a local site of production after, for example, β_2-adrenoceptor stimulation is very rapid and encompasses the whole cell (Figure 2a; see Ref. [29] for references). In cardiac myocytes, receptors such as the β_1-adrenoceptor produce generalized signals, whereas others such as the β_2-adrenoceptor (Figure 2b) signal locally (see Ref. [72] for an overview). A striated micropattern of cAMP gradients has been observed in neonatal cardiac myocytes with a genetically encoded PKA-based indicator, but it is not yet clear whether this striated pattern is related to striated targeting of the sensor by A-kinase-anchoring proteins [73]. Although the formation of these microdomains was attributed to the high activity of the phosphodiesterases PDE3 and PDE4 [73,74], the localized nature of the β_2-receptor cAMP signal was not abolished by phosphodiesterase inhibition [75]. Instead, formation of the microdomains might be due to a different localization of the receptors responsible [76]. Lastly, distinct kinetics of cAMP signals in different cellular compartments has been reported by cAMP imaging of both the submembrane and the nuclear space in transfected HEK293 cells [30]. Taken together, these studies indicate that signaling from GPCRs to cAMP might be encoded both in space and in time.

Summary and outlook

Optical techniques using luminescence and fluorescence have opened new avenues of research into signaling by G proteins and their receptors. They allow us views of the mechanisms of GPCR signaling, the kinetics of the individual steps, and the spatial and temporal patterns of signaling. In the future, this research will diverge in various directions. Mechanistic studies will aim to develop and use smaller labels to understand the molecular events that take place during GPCR signaling, and to elucidate the nature of signaling complexes. Studies on isolated cells and on cell populations will try to identify the codes of spatially and temporally confined signals and to address the issue of how such gradients are shaped. Finally, new imaging techniques that can penetrate deeper into tissues will be used to illustrate how GPCR signaling occurs *in vivo*.

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