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Localised GPCR signalling as revealed by FRET biosensors

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Förster resonance energy transfer (FRET) biosensors have provided much evidence for compartmentalised signalling following activation of G protein-coupled receptors (GPCRs). This localised signalling occurs within distinct plasma membrane microdomains and at sub cellular locations including endosomes, mitochondria, Golgi and the nucleus. Notable advances linking compartmentalisation to physiology have been made in two major areas: linking compartmentalised cAMP production by the β_2-adrenoceptor to excitation-contraction coupling in the heart; and selectively antagonising GPCRs within early endosomes to provide more efficacious inhibition of pain transmission. Important technological advances are also highlighted, including various approaches for the local activation of receptors, and the rational design of a FRET biosensor with a functional affinity that is not affected by the addition of a targeting sequence.

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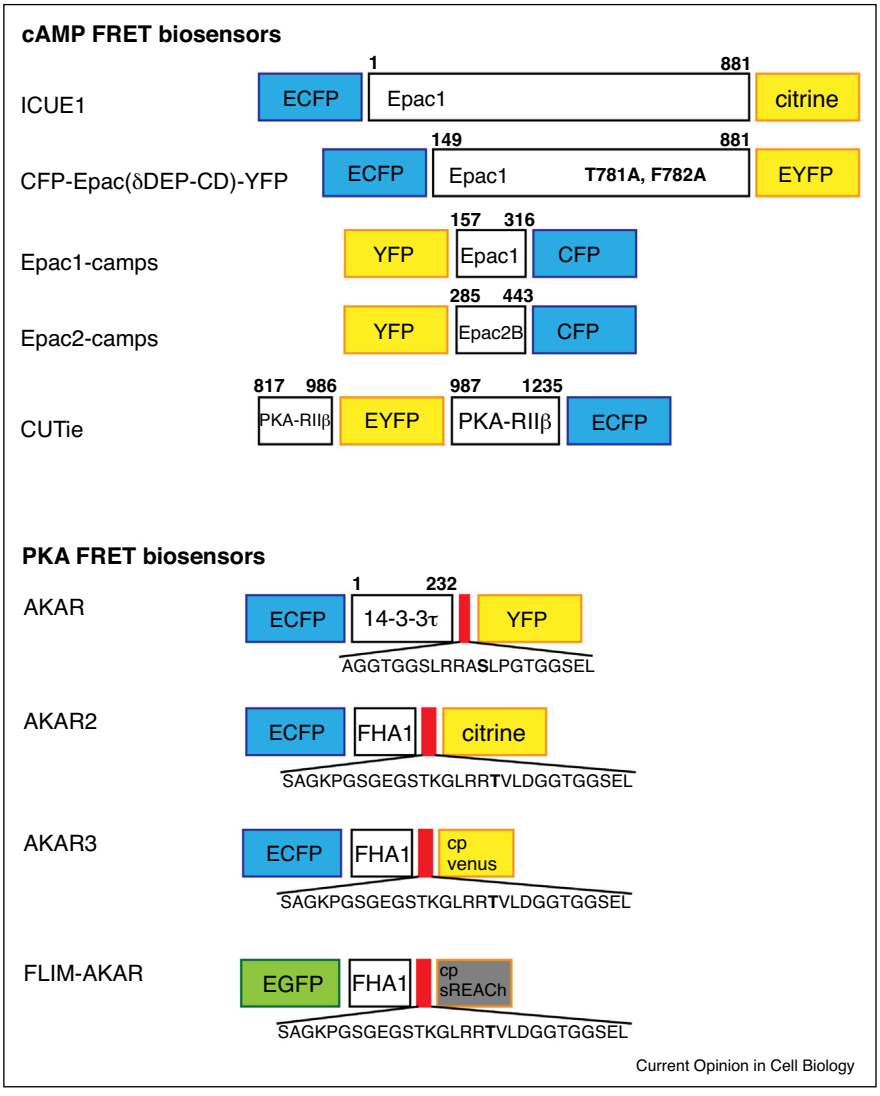
Introduction

Compartmentalised signalling — the regulation of signalling in space and time — is a mechanism for cells to distinguish between the activation of distinct receptors to generate highly-specific responses (reviewed in Ref. [1,2]). Since the first reports of the use of intramolecular FRET biosensors to monitor compartmentalised G protein-coupled receptor (GPCR) signalling [3–5], their use has rapidly expanded, revealing that many active GPCRs can also be found in discrete sub cellular compartments (reviewed in Refs. [6,7•]). Here, the evidence for localised GPCR signalling as revealed by FRET biosensors is discussed with a focus on reports in the last two years. Compartmentalisation of cAMP remains the focus of most studies. For example, many elegant studies have clearly demonstrated compartmentalisation of cAMP stimulated by the β_2-adrenoceptor in cardiac myocytes, and the loss of this in heart failure. This focus on cAMP compartmentalisation is likely due to two main factors: first, that $G\alpha_s$ and $G\alpha_{i/o}$-coupled receptors (which account for two thirds of all GPCRs with approved drugs [8]) have been classically defined by their ability to stimulate or inhibit cAMP levels, respectively; and second, there are many well-characterised FRET biosensors available to detect cAMP (Figure 1) that have been targeted to many sub cellular locations (Figure 2). Importantly, the challenge to link compartmentalised GPCR signalling with major physiological and pathophysiological processes has been addressed with advances in two areas: compartmentalised cAMP signalling of the β_2-adrenoceptor has been linked to excitation-contraction coupling of cardiac myocytes; and inhibition of endosomal signalling of the tachykinin NK_1 receptor, calcitonin CGRP receptor and the proteinase-activated receptor, PAR2, has been demonstrated to increase therapeutic efficacy of receptor antagonists. Finally, some elegant solutions to the limitations of the use of FRET biosensors are highlighted.

Localised GPCR signalling from distinct plasma membrane microdomains

The classic example of GPCR signal compartmentalisation is the control of cAMP signalling in cardiac myocytes; these cells express β-adrenoceptors and prostanoid receptors, all of which increase cAMP (reviewed in Ref. [9]). However, the physiological effects of activation of each of the receptors are different, and this has been attributed to compartmentalisation of the cAMP signal (reviewed in Ref. [9]). Compartmentalisation of cAMP has now been directly demonstrated using a cAMP FRET biosensor (Epac2-camps [3]) targeted to lipid-rich (Epac2-MyrPalm [10]) versus non-lipid-rich (Epac2-CAAX [10]) domains of the plasma membrane. Activation of cardiac myocytes or airway smooth muscle cells with a β-adrenoceptor agonist caused a similar amount of cAMP in both plasma membrane domains, whereas activation of a prostanoid receptor caused a larger cAMP increase in non-lipid-rich plasma membrane domains [11,12]. In the airway smooth muscle cells, the increase in prostanoid EP_2 receptor-stimulated cAMP within non-lipid-rich membrane domains corresponded with an increase in nuclear (detected by Epac2-NLS [11]), but not cytosolic cAMP [11]. Interestingly, in airway smooth muscle cells, the Epac2-CAAX biosensor was more concentrated around the nucleus suggesting a

Figure 1

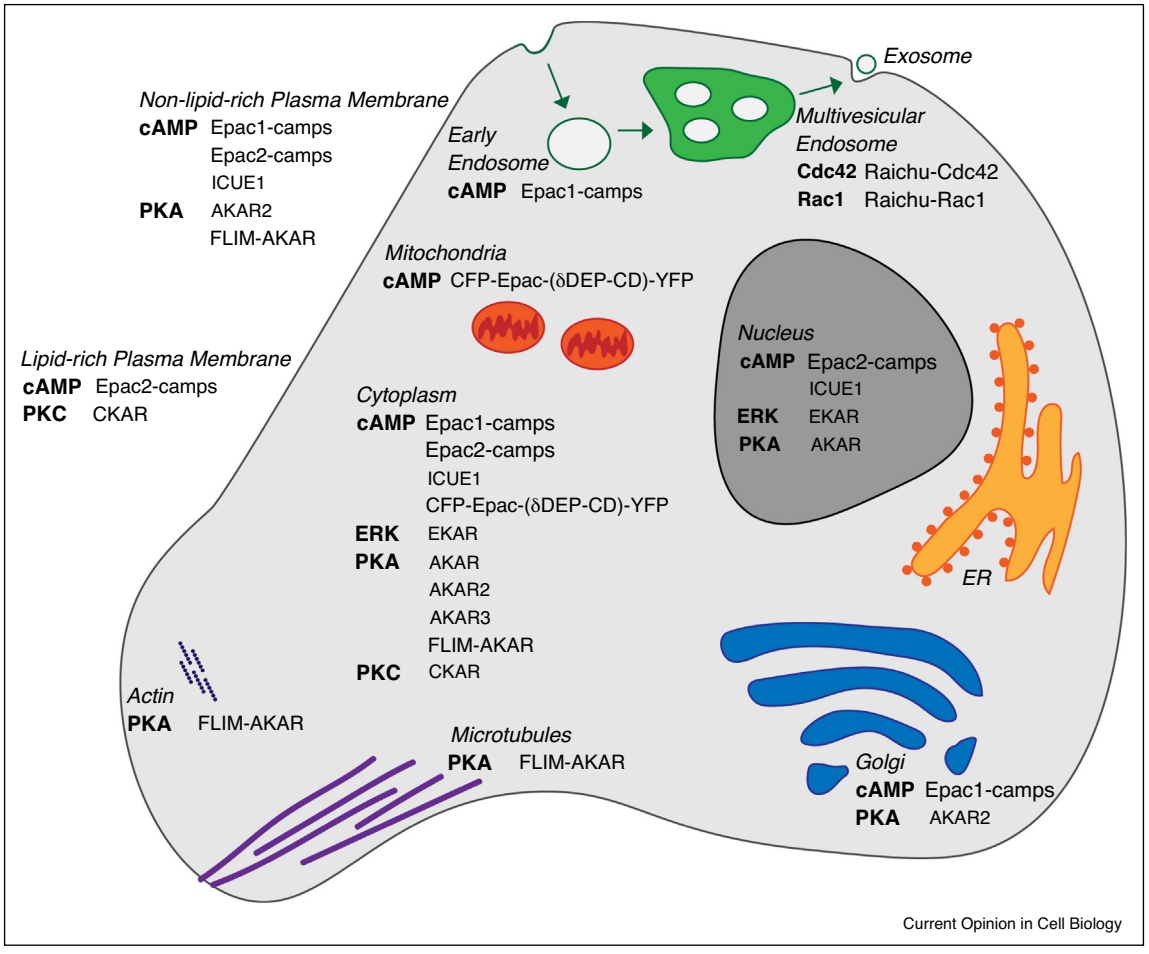


FRET biosensors for the detection of cAMP and PKA activity. While there are FRET biosensors available that detect many intracellular signalling molecules, those that detect changes in cAMP or PKA activity are the most widely developed and used for the study of localised GPCR signalling. The domain layouts for the various cAMP and PKA activity FRET biosensors used in the papers highlighted in this review are shown. FRET biosensors for cAMP include ICUE1 [5], CFP-Epac(δDEP-CD)-YFP [4], Epac1-camps [3], Epac2-camps [3] and CUTie [50••]. FRET biosensors for PKA include AKAR [36], AKAR2 [43], AKAR3 [30] and FLIM-AKAR [37••]. For the cAMP FRET biosensors, the proportion of the protein sequence included in the cAMP binding domain is indicated by the residue numbers above the white boxes. Any mutations within the primary sequence of these domains are indicated in bold. For the PKA FRET biosensors, the PKA phosphorylation target sequence (red box) is shown in full below the biosensor, and the proportion of the protein sequence included in the domain that interacts with the phosphorylated target sequence (white box) is indicated by the residue numbers above. Abbreviations: cp, circularly permuted; FHA1, forkhead associated domain 1 from Rad53p (residues 22–162); sREACh, a dark yellow fluorescent protein (quenching acceptor).

close association between non-lipid-rich plasma membrane domains and the perinuclear space [11].

In addition to signalling localised to different plasma membrane domains, the movement of GPCRs within the plasma membrane can affect the spatial and temporal control of compartmentalised signals further into the cell. Decades of research have demonstrated differential regulation and trafficking of the mu-opioid receptor (MOR) dependent on the activating ligand (reviewed in Ref. [13]). The prototypical analgesic, morphine, produces a distinct pattern of receptor phosphorylation and signalling, compared to other opioid ligands such as DAMGO [14•,15]. By using ERK and PKC biosensors (EKAR [16] or CKAR [17], respectively) targeted to the plasma membrane (pmCKAR [17]), cytosol (cytoEKAR,

Figure 2



Sub cellular targeting of FRET biosensors. Many FRET biosensors, but particularly those that detect changes in cAMP or the activity of PKA, have been directed to sub cellular locations, typically by the addition of a targeting motif to the N- or C-terminus of the biosensor. This cartoon shows the FRET biosensors that have been targeted to distinct sub cellular locations, with a focus on the research highlighted in this review. cAMP sensors have been targeted as follows: Epac1-camps [3] to non-lipid-rich plasma membrane (Epac1-CAAX [44]), early endosomes (Epac1-FYVE [32]) and Golgi (Epac1-GRIP [42]); Epac2-camps [3] to non-lipid-rich plasma membrane (Epac2-CAAX [10]), lipid-rich plasma membrane (Epac2-MyrPalm [10]) and nucleus (Epac2-NLS [11]); ICUE1 [5] to non-lipid-rich plasma membrane (pm ICUE1 [5]) and nucleus (NLS-ICUE1 [5]); CFP-Epac-(δDEP-CD)-YFP [4] to the mitochondria (4mtH30 [39]). PKA sensors have been targeted as follows: AKAR [36] to the nucleus (NLS-AKAR [5]); AKAR2 [43] to non-lipid-rich plasma membrane (AKAR2-CAAX [42]) and Golgi (AKAR2-GRIP [42]); AKAR3 [30] has been used to detect cytosolic PKA; and FLIM-AKAR [37••] to non-lipid-rich plasma membrane (tAKARγ [37••]), actin (tAKARδ and tAKARε [37••]) and microtubules (tAKARα [37••]). A PKC biosensor [17] has been targeted to lipid-rich plasma membrane (pmCKAR [17]), and an ERK biosensor [16] to the nucleus (nucEKAR [16]). The activity of Cdc42 (Raichu-Cdc42 [34]) and Rac1 (Raichu-Rac1 [34]) was measured in the immediate vicinity of multi-vesicular endosomes by co-labelling [35•].

[16]) or the nucleus (nucEKAR, [16]), we demonstrated transient increases in cytosolic and nuclear ERK in response to DAMGO that preceded receptor internalisation and were due to a lateral reorganisation of the MOR at the plasma membrane [14•]. In contrast, morphine caused a sustained increase in plasma membrane PKC and cytosolic ERK, due to a restricted receptor distribution [14•]. Inhibition of factors constraining MOR location (Gβγ-PKC-phosphorylation pathway) allowed morphine to redistribute the receptor and activate transient ERK activity in the cytosol and nucleus, without receptor internalisation [14•]. Using the same FRET biosensors, the magnitude of ERK activity within the cytosol and nucleus was recently linked to the lifetime of MOR/β-arrestin complexes at the cell surface [18].

Localised signalling controlled by GPCRs at intracellular locations

There are many examples of compartmentalised signalling detected by FRET biosensors that have been linked to GPCR internalisation using endocytic inhibitors and/or by correlating the timing of this signalling with receptor

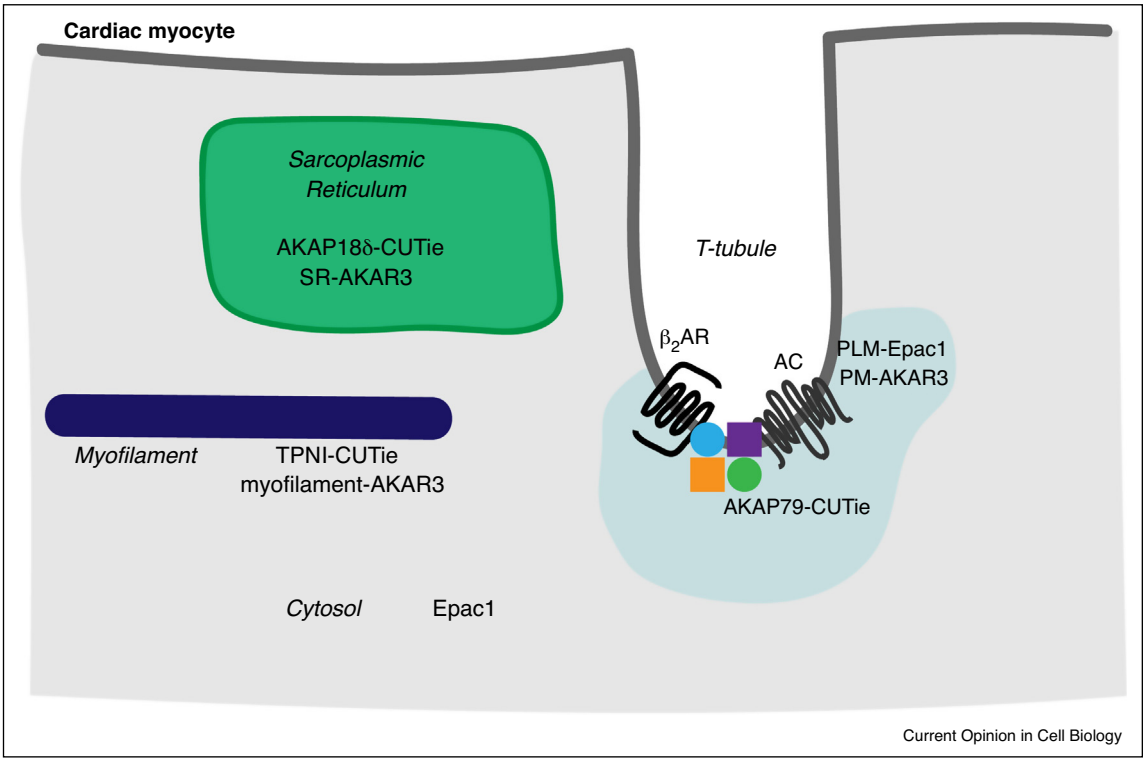
internalisation [19–24]. Other elegant studies have combined FRET biosensors with detailed image analysis to show localised Cdc42 activity within invadosomes [25], and distinct control of GPCR signalling in the cilia, dendrites, synaptic boutons and soma of neurons [26•,27,28•]. Of particular note are a number of studies that directly measured compartmentalised signals at their intracellular location and/or linked this signalling to a physiological outcome (see below and Figure 3).

Endosomes

The parathyroid hormone PTH1 receptor was one of the first GPCRs shown to be active within endosomes [29]. The use of biosensors for cAMP and PKA targeted to the cytosol (Epac1-camps [3] and AKAR3 [30], respectively) and nucleus (NLS-ICUE1 and NLS-AKAR [5], respectively), has shown that co-activation of $G\alpha_{i/o}$-coupled GPCRs within endosomes containing the PTH1 receptor causes a synergistic increase in the magnitude and duration of the cAMP and PKA signal [31]. The endosomally produced cAMP diffused to the nucleus where it caused an additional increase in PKA activity, associated with gene transcription and the formation of mineralised nodules in osteoblast-like ROS17/2.8 cells [31].

A sustained increase in cAMP linked to receptor internalisation was also reported for the gastric inhibitory polypeptide (GIP) receptor (using cytosolic Epac1-camps [3] and AKAR3 [30]) and following activation of the leutenising hormone (LH) receptor in ovarian follicles isolated from mice transgenic for Epac1-camps [32,33]. In both cases, the contribution of the internalised receptor to the cAMP/PKA response was most evident when assessing the reversibility of signalling: the response was only reversible following ligand washout after inhibition of endocytosis [32,33]. While cAMP produced by the internalised GIP receptor was subsequently localised to early endosomes (using a targeted cAMP biosensor, Epac1-FYVE) [32], sustained cAMP from internalised LH receptors was linked to the induction of meiosis resumption [33].

Figure 3



Localised β_2-adrenoceptor signalling in cardiac myocytes. The localisation of cAMP signalling to the T-tubules following activation of the β_2-adrenoceptor in cardiac myocytes is well defined. Recent elegant studies have utilised either cAMP (Epac1-camps [3] and CUTie [50••]) or PKA (AKAR3 [30]) FRET biosensors targeted to specific sub cellular domains or protein complexes (represented at the β_2-adrenoceptor by coloured circles and squares) to explain how this compartmentalisation of signalling (light blue area) facilitates efficient excitation-contraction coupling. Epac1-camps [3] was targeted to phospholemman (PLM-Epac1 [49]); AKAR3 [30] was targeted to the plasma membrane (PM-AKAR3 [30]), sarcoplasmic reticulum (SR-AKAR3 [51]), and myofilaments (myofilament-AKAR3 [52]); CUTie [50••] was targeted to the β_2-adrenoceptor at the plasma membrane (AKAP79-CUTie [50••]), sarcoplasmic reticulum (AKAP18δ-CUTie [50••]) and myofilaments (TPNI-CUTie [50••]). Abbreviations: AC, adenylyl cyclase; AKAP, A-kinase anchoring protein; β_2AR, β_2-adrenoceptor; Epac1, Epac1-camps; PLM, phospholemman; PM, plasma membrane; SR, sarcoplasmic reticulum; TPNI, troponin I.

Mutilvesicular endosomes

Localised GPCR signalling has been linked to exosome release; activation of the sphingosine 1-phosphate receptor, $S1P_1$, by uncaging S1P caused increased Cdc42 and Rac1 activity (Raichu-Cdc42 and Raichu-Rac1 [34], respectively) at the multivesicular endosome [35•]. This led to increased actin nucleation, which the authors contend provides rails for cargoes during sorting into exosomal intralumenal vesicles [35•].

Microtubules

A cytosolic PKA biosensor (AKAR [36]) optimised for two-photon fluorescence lifetime imaging microscopy (FLIM-AKAR) was targeted to sub cellular regions including the microtubules (tAKARα), actin cytoskeleton (tAKARδ and tAKARε), post-synaptic density (tAKARζ) or plasma membrane (tAKARγ) [37••]. Norepinephrine activation of CA1 neurons caused a 2.7-fold greater increase in PKA activity at the microtubules compared to bulk cytosol, the actin cytoskeleton or the plasma membrane [37••]. This localisation of norepinephrine-stimulated PKA activity to microtubules was also found in CA3 neurons in cultured hippocampal slices, apical dendrites of layer 2/3 pyramidal neurons in somatosensory cortex slices, and *in vivo* imaging of barrel and motor cortices in awake mice [37••]. Microtubule-localised PKA activity was linked to CA1 neuron slow afterhyperpolarisation and the degree of wakefulness and locomotor activity in whole animals [37••].

Mitochondria

In N2a cells, the melatonin MT_1 receptor was detected in the mitochondria, together with $G\alpha_i$ and adenylyl cyclase (AC) 5 [38]. Using the cAMP biosensors ICUE1 [5] or CFP-Epac(δDEP-CD)-YFP [4] targeted to the plasma membrane (pm ICUE1 [5]) or to the outer mitochondrial membrane (4mtH30 [39]), respectively, the melatonin-mediated inhibition of forksolin-stimulated cAMP was stronger at the mitochondria compared to the plasma membrane. In contrast, a cell impermeant melatonin receptor ligand, ICOA-13, inhibited forskolin-stimulated cAMP at the plasma membrane, but had little effect on the cAMP produced at the mitochondria [38,40].

Golgi

The thyrotropin-releasing hormone, TRH_1, receptor was one of the first GPCRs identified to show sustained cAMP production following receptor internalisation [41]. However, the TRH_1 receptor within early endosomes is not associated with G protein recruitment or cAMP signalling [42]. Rather, the TRH_1 receptor accumulates with $G\alpha_s$ in a retromer-coated compartment that mediates retrograde trafficking from endosomes to the Golgi [42]. To confirm the Golgi TRH_1 receptor was active, cytosolic cAMP (Epac1-camps [3]) and PKA (AKAR2 [43]) biosensors were targeted to the plasma membrane (Epac1/AKAR2-CAAX [42,44]) and the Golgi (Epac1/AKAR2-GRIP [42]). Activation of the endogenous TRH_1 receptor in mouse primary thyroid cells caused a rapid increase in cAMP and PKA at the plasma membrane [42]. In contrast, TRH addition caused a slower and delayed (approximately 8 min) increase in cAMP and PKA activity within the Golgi, which was abolished following inhibition of endocytosis [42]. Importantly, the increase in cAMP/PKA activity within the Golgi was linked to the transcription of specific genes [42].

Compartmentalised β-adrenoceptor signalling in cardiac myocytes

The challenge for the study of compartmentalised signalling is to directly demonstrate its relevance at the level of the whole organism or during pathogenesis. Localised cAMP produced by the β_2-adrenoceptor has been well-studied using FRET biosensors in cardiac myocytes where it is important for excitation-contraction coupling (reviewed in Ref. [45]). In these cells, the assembly of a β_2-adrenoceptor-protein complex at the T-tubules restricts cAMP to a very localised region; this organisation is disrupted in heart failure models, resulting in diffuse production of cAMP throughout the cell [46]. T-tubules contain sodium-potassium ATPase (NKA), which plays a key role in excitation-contraction coupling, and forms a stable complex with phospholemman [47,48]. The cAMP biosensor, Epac1-camps [3], was targeted to these microdomains using phospholemman (PLM-Epac1) [49] (Figure 3). Activation of the β_2-adrenoceptor selectively elevated cAMP within NKA/PLM microdomains, with a 26-fold increase in cAMP concentration compared to the bulk cytosol (defined by cytosolic Epac1-camps) [49]. In heart failure models, the compartmentation of β_2-adrenoceptor signalling was lost, with only a 2.4-fold increase in cAMP concentration within NKA/PLM microdomains versus bulk cytosol [49].

Biosensors for cAMP and PKA have been further targeted to three protein complexes that have important roles in excitation-contraction coupling (Figure 3): the AKAP18δ/SERCA/PLB complex localised to the sarcoplasmic reticulum which regulates Ca^{2+} reuptake (AKAP18δ-CUTie [50••] and SR-AKAR3 [51]), the troponin TPN1/TPNT/TPNC complex localised to myofilaments that regulates their Ca^{2+} sensitivity (TPNI-CUTie [50••] and myofilament-AKAR3 [52]), and the AKAP79/β_2-adrenoceptor/AC/LTCC complex at the plasma membrane that regulates cAMP and Ca^{2+} influx (AKAP79-CUTie [50••] and PM-AKAR3 [30]). Isoprenaline stimulation caused a much faster and larger increase in cAMP detected by AKAP79-CUTie (plasma membrane) and AKAP18δ-CUTie (sarcoplasmic reticulum), compared to TPNI-CUTie (myofilaments) [50••]. Consistent with this, a much greater β_2-adrenoceptor-stimulated PKA activity was recorded at the plasma membrane versus sarcoplasmic reticulum or myofilaments [52]. This localised activity can be related to the cellular consequences of PKA

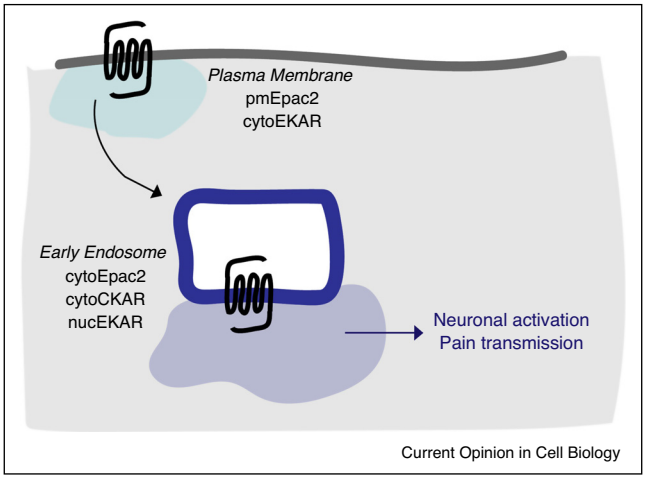
phosphorylation of the target complexes: PKA phosphorylation of phospholambin B (AKAP18δ-CUTie) and L-type Ca^{2+} channels (AKAP79-CUTie) causes increased Ca^{2+} transients and contraction, whereas PKA phosphorylation of troponin (TPNI-CUTie) decreases myofilament Ca^{2+} sensitivity and limits contraction [50••]. Therefore, for β_2-adrenoceptor-cAMP to cause efficient increases in contractility, there needs to be increased cAMP at AKAP18δ/SERCA/PLB and AKAP79/β_2-adrenoceptor/AC/LTCC complexes, but less cAMP at the troponin TPN1/TPNT/TPNC complex. Consistent with this, there was an increase in β_2-adrenoceptor-stimulated PKA activity at the myofilaments in cardiac myocytes from heart failure models [52].

Notably, a recent study using cAMP biosensors targeted to the cytosol (Epac2-camps [3]), plasma membrane (pmEpac2 [53]), and to PKA-RII subunits (RII-Epac2 [54••]) in isolated cells and whole hearts, has revealed an anatomical relevance of cAMP compartmentation. Cardiomyocytes from the apex of the heart demonstrated a decreased membrane complexity (less T-tubules, less caveolae), and little compartmentalisation of β_2-adrenoceptor-stimulated cAMP [54••]. As a result, β_2-adrenoceptor-stimulated cAMP activated PKA in multiple compartments to efficiently activate excitation-contraction coupling, and apical cardiomyocytes displayed greater contraction and faster relaxation [54••]. In contrast, local stimulation of the β_2-adrenoceptor in the T-tubules of basally-derived cardiomyocyes led to cAMP increases only within the same domain; these cells displayed less shortening in response to β_2-adrenoceptor stimulation [54••]. Remarkably, these findings were replicated in the basal and apical regions of hearts isolated from pmEpac2 transgenic mice [54••]. The authors propose that the anatomical differences in β_2-adrenoceptor stimulation may be important for the selective enhancement of apical function without associated left ventricular outflow track obstruction, making it an 'ideal reservoir of contractile force . . . in stressful situations' [54••].

Localised signalling from GPCRs in endosomes regulates pain transmission

We recently demonstrated the therapeutic relevance of targeting localised GPCR signalling within endosomes to increase the therapeutic efficacy of antagonists in models of pain [55••,56,57] (Figure 4). For the tachykinin NK_1 receptor, activation by substance P caused a transient increase in plasma membrane localised cAMP and cytosolic ERK, dependent on receptor localisation at the plasma membrane [55••]. After receptor internalisation to early endosomes, the NK_1 receptor instead caused a sustained increase in cytosolic cAMP, cytosolic PKC activity and nuclear ERK activity [55••]. For the calcitonin CGRP receptor, while all signals had the same sustained temporal profile, activation of cytosolic ERK was dependent on a plasma membrane localised receptor, whereas activation of cytosolic PKC and nuclear ERK was dependent on internalisation of the receptor to endosomes [56]. Specific blockade of receptors within endosomes, using cholestenol-conjugated antagonists which accumulate in the endosomal network, abolished nuclear ERK and decreased pain transmission [55••,56]. This was particularly effective when two of the cholestenol-conjugated antagonists (spantide-cholestanol and $CGRP_{8\text{-}37}$-cholestanol targeting the NK_1 and CGRP receptors, respectively) were co-administered, alleviating the pain response by 50% [56].

Figure 4



Localised signalling from GPCRs in endosomes controls pain transmission. Localised signalling stimulated by the activated NK_1 receptor [55••], CGRP receptor [56] and PAR2 [57] within early endosomes facilitates neuronal activation and pain transmission. This endosomal signalling (purple shading) is distinct from that activated by plasma membrane localised receptors (blue shading). Signalling from receptors within endosomes can be selectively blocked by antagonists that are targeted to the endosomal network by the addition of a cholestanol moiety.

There is also evidence for a contribution of localised GPCR signalling to the chronic pain associated with irritable bowel syndrome (IBS). In this case, sustained hyperexcitability of dorsal root ganglion neurons due to activation of the proteinase-activated receptor, PAR2, by trypsin was dependent on signalling from endosomes [57]. Trypsin caused increases in cAMP, PKC and ERK activity, all of which were decreased following inhibition of endocytosis [57]. Selective inhibition of PAR2 within endosomes using cholestenol-conjugation of an antagonist decreased neuronal excitability in response to supernatants from biopsies of colonic mucosa from IBS subjects, but not healthy controls [57].

Perspective

FRET biosensors have been an extremely powerful method with which to unravel the importance of

compartmentalised signalling in the control of cellular processes. Much of the evidence for signalling of internalised GPCRs initially relied on measuring a change in signal following the inhibition of endocytosis by chemical means or by the over expression of dominant negative proteins essential to this process. This has been an undoubtedly effective approach; however, it will also impact on the cycling of many proteins within the cell. Some very elegant alternatives have emerged recently. For example, receptors within specific microdomains can be selectively activated by uncaging ligands and then calculating the FRET signal only in regions that have co-localisation with a domain marker (e.g. in Ref. [35•]), or by the local application of drugs to cellular landmarks using iontophoresis [28•,37••] and scanning ion conductance microscopy nanopipettes [54••]. Alternatively, high resolution image analysis strategies such as analysing the change in FRET signals across the cellular depth, have successfully illustrated compartmentalised signalling in the absence of cell perturbation [26•,27,28•]. Finally, there is a growing list of sub cellular locations where the FRET biosensors have been targeted by the addition of localisation motifs. This provides another, perhaps more 'high-throughput', way to directly measure compartmentalised signals without perturbing cells. However, the addition of targeting sequences often alters the sensitivity of the original biosensor for its signalling mediator [58]. To circumvent this, the FRET biosensors must be calibrated, to convert the FRET signal to an absolute concentration of intracellular effector (e.g. in Ref. [49]). A very elegant alternative approach was recently described; a novel cAMP FRET biosensor was rationally designed to have a dynamic range that is unaffected by differential targeting [50••]. This biosensor, termed CUTie (cAMP universal tag for imaging experiments), was successfully targeted to six locations without a change in sensitivity for cAMP. This allows the direct comparison of FRET signals within different compartments without conversion to absolute concentrations of effectors or without comparison to a reference ligand. To be able to directly compare the same FRET biosensor at multiple sub cellular locations would be a wonderful way to quickly assess localised signalling of GPCRs.

The next few years are likely to continue to provide more evidence of compartmentalisation of GPCR signalling. As more studies start to assess the effect of GPCR localisation on signalling effectors more distal to receptor activation than cAMP, such as the activation of small G proteins or kinases, we may also observe greater compartment-based differences in the signals. The pinnacle is still to clearly show that compartmentalised signals mediate distinct whole system responses, and that there is therapeutic utility in selectively inhibiting or enhancing a signal in one compartment over signals from other cellular regions. The emergence of more multi-disciplinary studies in pathophysiologically relevant systems will clearly demonstrate the therapeutic gain in efficacy that can be obtained if GPCR drugs are made to specifically target signals from discrete locations.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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