Kinetic diversity in G-protein-coupled receptor signalling

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The majority of intracellular signalling cascades in higher eukaryotes are initiated by GPCRs (G-protein-coupled receptors). Hundreds of GPCRs signal through a handful of trimeric G-proteins, raising the issue of signal specificity. In the present paper, we illustrate a simple kinetic model of G-protein signalling. This model shows that stable production of significant amounts of free $G_{\alpha}^{\text{GTP}}$ (GTP-bound $G_{\alpha}$ subunit) and $\beta\gamma$ is only one of multiple modes of behaviour of the G-protein system upon activation. Other modes, previously uncharacterized, are sustained production of $\beta\gamma$ without significant levels of $G_{\alpha}^{\text{GTP}}$ and transient production of $G_{\alpha}^{\text{GTP}}$ with sustained $\beta\gamma$. The system can flip between different modes upon changes in conditions. This model demonstrates further that the negative feedback of receptor uncoupling or internalization, when combined with a positive feedback within the G-protein cycle, under a broad range of conditions results not in termination of the response but in relaxed oscillations in GPCR signalling. This variety of G-protein responses may serve to encode signal specificity in GPCR signal transduction.

Key words: G-protein-coupled receptor (GPCR), kinetics, modelling, oscillation, transient response, trimeric G-protein.

INTRODUCTION

GPCRs (G-protein-coupled receptors) constitute one of the biggest protein families in animals: more than 800 GPCR genes have been identified in the human genome, far outnumbering genes for other receptor types [1]. GPCRs recognize a vast array of extracellular signals, ranging from quanta of light to small nucleotide molecules and peptides to huge glycoproteins. They govern a multitude of cellular and organismal responses, such as receiving of sensory information, neurotransmission, immunity and development. More than half of all marketed therapeutic agents target GPCRs [2]. Our understanding of the mechanisms of GPCR functioning is crucial for biology and medicine.

GPCRs signal through activation of trimeric G-proteins. These proteins in the resting state exist as heterotrimers of $\alpha$, $\beta$, and $\gamma$ subunits, where the $\alpha$-subunit is bound to GDP. The $G_{\alpha}^{\text{GDP}}\beta\gamma$ complex can associate with GPCRs. Biochemical analysis provides a wealth of information about the G-protein cycle [3–5]. Activated GPCRs function as GEFs (guanine-nucleotide-exchange factors) for trimeric G-proteins, catalysing the exchange of GDP on Gq for GTP. This leads to dissociation of the complex into $G_{\alpha}^{\text{GTP}}$ and $\beta\gamma$, which can signal independently to various downstream effectors. Over time, GTP on Gq is hydrolysed back to GDP by the intrinsic GTPase activity of Gq, with the aid of GAPs (GTPase-activating proteins). GAP activity can be exerted by some effectors, such as PLC$\beta$ (phospholipase C$\beta$), or by specialized RGS (regulator of G-protein-signalling) proteins [5]. Upon conversion into the $G_{\alpha}^{\text{GDP}}$ state, Gq can bind $\beta\gamma$ and reconstitute the original complex, ready for a new round of signalling.

A generally accepted view of GPCR signalling is that receptor activation leads to a large increase in steady-state concentrations of free $G_{\alpha}^{\text{GTP}}$ and $\beta\gamma$, proportional to the ‘strength’ of GPCR activation. High concentrations of free $G_{\alpha}^{\text{GTP}}$ and $\beta\gamma$ trigger intracellular signalling cascades, and fall back as a result of removal of the ligand from the extracellular space (signal termination), removal of the receptor from the cell surface (internalization) or covalent uncoupling of the receptor from G-proteins.

This view is based on biochemical observations, such as the stable rise of GTP incorporation into and hydrolysis by G-proteins upon receptor stimulation [3], as well as stable receptor-induced dissociation of $\beta\gamma$ from Gq in cell populations, terminated only by ligand washout [6] or receptor internalization [7]. These observations have been reproduced using kinetic simulations [7–11].

The uniformity in G-protein activation by GPCRs raises the issue of specificity in GPCR signalling. Indeed, hundreds of GPCRs have to signal through a limited set of G-proteins: only 16 genes for Gq subunits exist in humans [12] (six in Drosophila [13]). This issue is aggravated further by the promiscuity in the GPCR signalling: receptors and effectors usually do not discriminate between different $\beta\gamma$ subunits [4]; moreover, receptors can activate G-proteins containing different Gq subunits [14], and genetic ablation of a Gq subunit often results in no or limited phenotypes, as other Gq subunits step up to fulfil the disrupted function [12].

In the present paper, we describe a novel kinetic model of trimeric G-protein signalling. Its simplicity is combined with careful examination of the reactions governing the G-protein cycle, as well as extensive parameter estimation from experimental data. This model challenges the conventional view of trimeric G-protein signalling and predicts a large variety of G-protein kinetic responses to receptor activation.

MATERIALS AND METHODS

Modelling equations

The kinetic model of the trimeric G-protein cycle (Figure 1) contains as variables concentrations of the trimeric G-protein complex ($[G_{\alpha}^{\text{GDP}}\beta\gamma]$) and its derivatives ($[\beta\gamma]$, $[G_{\alpha}^{\text{GTP}}]$ and $[G_{\alpha}^{\text{GDP}}]$), and as constants concentrations of the activated GPCR ($[R_{\text{c}}]$) and the GAP ($[\text{GAP}]$).

Changes in molar concentrations of the components of the cycle are described through rates of production and destruction of the respective components; it becomes apparent that only three rates (designated as $V_1$, $V_2$ and $V_3$) govern the behaviour of this simple

Abbreviations used: GAP, GTPase-activating protein; GEF, guanine-nucleotide-exchange factor; GPCR, G-protein-coupled receptor; GRK, GPCR kinase.

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Figure 1  Trimeric G-protein cycle

The cycle contains four dependent variables ([Gα2·βγ], [Gα·βγ], [Gα·βγ] and [Gα2·βγ]) and two independent variables (Rc* (activated receptor) and GAP). Three reactions (labelled 1–3 respectively) govern the cycle: association of βγ and Gα·βγ into the trimeric complex, Rc*-catalysed dissociation of the trimeric complex into βγ and Gα2·βγ, and Gα2·βγ-catalysed hydrolysis of GTP on Gα2·βγ with formation of Gα·βγ. The rates of these reactions, V1, V2 and V3, are described in eqns (2)–(4) in the Materials and methods section.

The cycle contains four dependent variables (Gα·βγ, Gα2·βγ, βγ and Gα2·βγ) and two independent variables (Rc* (activated receptor) and GAP). The Michaelis–Menten rate equations have to be used: $V = \frac{k_{\text{cat}} [G\alpha^{\text{GDP}}][\beta\gamma]}{K_M + [G\alpha^{\text{GDP}}][\beta\gamma]}$ and Gα2·βγ-catalysed hydrolysis of GTP on Gα2·βγ with formation of Gα·βγ. The rates of these reactions, V1, V2 and V3, are described in eqns (2)–(4) in the Materials and methods section.

where $V_1$ is the rate of re-association of the trimeric G-protein complex, $V_2$ is the rate of dissociation of the trimeric complex, and $V_3$ is the rate of hydrolysis of GTP on Gα. The respective reactions are depicted as ①, ② and ⑤ in Figure 1.

As one Gα subunit and one βγ heterodimer make one trimeric complex, and their association is not enzyme-catalysed, the rate $V_1$ is described simply as:

$$V_1 = k_{\text{ass}}[G\alpha^{\text{GDP}}][\beta\gamma] = k_{\text{ass}} n_1 n_2,$$

where $k_{\text{ass}}$ is the association rate constant. In contrast, reactions described by rates $V_2$ and $V_3$ are enzymatic, with activated receptor (Rc*) catalysing dissociation of the trimeric complex, and GAP catalysing the GTP hydrolysis by Gα (Figure 1). Thus the Michaelis–Menten rate equations have to be used:

$$V_2 = \frac{k_{\text{diss}}[G\alpha^{\text{GDP}}][\beta\gamma][Rc^*]}{K_2 + [G\alpha^{\text{GDP}}][\beta\gamma]} = k_2 \frac{n_1}{K_2 + n_1}$$

where $k_{\text{diss}}$ is the dissociation rate constant, $K_2$ is the Michaelis–Menten constant for the receptor-catalysed dissociation of the trimeric complex, and $k_2 = k_{\text{diss}}[Rc^*]$. Similarly:

$$V_3 = \frac{k_{\text{hyd}}[G\alpha^{\text{GTP}}][\text{GAP}]}{K_3 + [G\alpha^{\text{GTP}}]} = k_3 \frac{n_3}{K_3 + n_3}$$

where $k_{\text{hyd}}$ is the hydrolysis rate constant, $K_3$ is the Michaelis–Menten constant for the GAP-accelerated GTP hydrolysis, and $k_3 = k_{\text{hyd}}[\text{GAP}].$

Mass conservation requires that:

$$[G\alpha^{\text{GDP}}][\beta\gamma] + [\beta\gamma] = n_1 + n_2 = M = \text{const}$$

$$[G\alpha^{\text{GTP}}] + [G\alpha^{\text{GDP}}] = n_3 + n_4 = n_2$$

where $M$ is the total (trimeric-complexed plus free) concentration of the G-protein.

Thus the four dependent differential equations (eqns 1a–1d) can be rewritten as a system of two independent differential equations:

$$\frac{d[G\alpha^{\text{GTP}}]}{dt} = \frac{dn_3}{dt} = k_2 \frac{M - n_2}{K_2 + (M - n_2)} - k_3 n_3$$

$$\frac{d[\beta\gamma]}{dt} = \frac{dn_1}{dt} - k_1 n_1 k_2 + (M - n_2) - k_3 n_3$$

having $[G\alpha^{\text{GTP}}]$ and $[\beta\gamma]$ as the two variables. After solving eqns (6a) and (6b), concentrations of $G\alpha^{\text{GDP}}[\beta\gamma]$ and $G\alpha^{\text{GDP}}$ are determined using eqns (5a) and (5b).

Alternative modelling equations (resulting in the same characteristics of the trimeric G-protein system) are given in the Supplementary Materials section at http://www.BiochemJ.org/bj/401/bj4010485add.htm.

Obtaining parameters for kinetic modelling

Published data (summarized in Supplementary Tables 2–5 at http://www.BiochemJ.org/bj/401/bj4010485add.htm) were used to obtain the rate constants and the Michaelis–Menten constants, as well as to find the range of trimeric G-protein, receptor and GAP intracellular concentrations. To convert the published data on the number of molecules per cell, cell volume estimates were used as shown in Supplementary Table 1 (http://www.BiochemJ.org/bj/401/bj4010485add.htm).

The obtained values (see Supplementary Table 2) show that the intracellular concentrations of Gα·G-proteins vary in the range 200 nM–3 μM, those of GPCRs vary in the range 1–500 nM, and those of GAPs vary in the range 10–300 nM.

It should be noted that local plasma membrane concentrations of the components of the trimeric G-protein cycle may be much higher than the total intracellular concentrations shown above. However, kinetic modelling at much higher concentrations reproduces the phenomena presented in the Results section, as with increased concentrations of the components of the cycle all three reactions speed up.

The $k_{\text{ass}}$ value for re-association of the trimeric complex from Gα·βγ and βγ varied in different experiments (see Supplementary Table 3); $k_{\text{diss}}$ for the trimeric G-protein G, using two different methods was found to be $4 \times 10^7 M^{-1} \cdot s^{-1}$ [15] or $0.7 \times 10^7 M^{-1} \cdot s^{-1}$ [16], with respective dissociation constant, $K_d = 100$ or 3 nM. Later $K_d$ measurements [17] yielded even lower value of 0.2 nM for Gα, and 17–27 nM for the Gα-G-proteins Gα and Gα. It is thus likely that the $k_{\text{ass}}$ value for Gα is fairly high (approx. $10^5 M^{-1} \cdot s^{-1}$), whereas other G-proteins such as Gα and Gα have an $k_{\text{ass}}$ value of approx. $10^5 M^{-1} \cdot s^{-1}$ or lower.

Published $k_{\text{ass}}$ values for receptor-driven trimeric G-protein dissociation vary widely (see Supplementary Table 4), from approx. 1 to 300 s$^{-1}$. The low values of $k_{\text{ass}}$ were obtained in reconstructed systems and are likely to be underestimations, while the extremely high values obtained for rhodopsin/transducin may not be transferable to other receptor/G-protein systems. Likely in vivo $k_{\text{ass}}$ values were estimated to be 20 s$^{-1}$ [18] and were varied in 5–25 s$^{-1}$ intervals for the present analysis.

From a general biochemical standpoint, the Michaelis–Menten constant for the receptor-driven trimeric G-protein dissociation ($K_2$ in eqn 3) is likely to be in the range of physiological G-protein concentration [19], which is indeed the case for transducin activation by rhodopsin [20]. Thus, although direct measurements for the Michaelis–Menten constant for other G-proteins are unavailable, we set $K_2$ at 500 nM.
GAPs were found to accelerate the GTP hydrolysis by orders of magnitude with a low Michaelis–Menten constant ($K_v$ in eqn 4) of approx. 2 nM [21]. Specific $K_{\text{M}}$ values differ depending on the Go–GAP pair (see Supplementary Table 5) and were varied in the $1–20$ s$^{-1}$ interval in the present modelling.

**Steady-state analysis**

Numerical solutions to the system of non-linear equations (eqn 6a and 6b) are shown in the Results section. Here we present approximate solutions allowing us to estimate analytically the steady-state concentrations of the trimeric G-protein components.

The trimeric G-protein system reaches the steady-state when the derivatives in eqns (6a) and (6b) become zero and the rates $V_1$, $V_2$ and $V_3$ become equal. Attempts to solve eqns (6a) and (6b) directly produce cumbersome equations of the fourth order. However, the Michaelis–Menten equations for the rates $V_2$ and $V_3$ eqns (3) and (4) approach constants at substrate concentrations greatly exceeding the Michaelis–Menten constant ($K_v$ and $K_β$ in eqns 3 and 4). This is easily the case for $V_2$, as $K_v$ is low (see above). So, we separately analysed two different steady-state situations: with high steady-state $[G\text{GTP}]$ ($>K_v$), and low steady-state $[G\text{GTP}]$ ($<K_v$). Under conditions of high $[G\text{GTP}]$, $V_3$ becomes:

$$V_3 = k_3 \frac{n_1}{K_v + n_3} \approx k_3 \tag{7}$$

Eqns (6a) and (6b) for the steady-state can now be rewritten as:

\[ \begin{align*}
{k_2 \frac{M - n_2}{K_2 + (M - n_2)}} &= k_1 n_2 (n_2 - n_1) \quad \text{(8a)} \\
{k_2 \frac{M - n_2}{K_2 + (M - n_2)}} &= k_i 
\end{align*} \]

Solving eqns (8a) and (8b) produces:

\[ \begin{align*}
[\beta \gamma] &= n_2 = M - k_1 \frac{K_v}{k_v - k_i} \quad \text{(9a)} \\
[\text{Gα}\text{GTP}] &= n_3 = M - k_i \frac{K_v}{k_v - k_i} - k_i M (k_v - k_i) - k_i K_v 
\end{align*} \]

Eqns (9a) and (9b) have been obtained by approximating $K_v = 0$. This approximation is only valid for the set of parameters resulting in high steady-state $[\text{Gα}\text{GTP}]$. In a different set of parameters, one or both solutions of eqns (9a) and (9b) can become ‘non-biological’, i.e. $<0$ or $>M$. It appears that if $n_2 > 0$ and $n_1 > 0$, the conditions $n_2 < M$ and $n_3 < M$ will apply. As a result, the following inequalities follow from eqns (9a) and (9b):

\[ \begin{align*}
{k_2 \frac{M}{K_2 + M}} &> k_i \quad \text{(10a)} \\
{k_i M^2} &> k_i \left[ \frac{M(k_v - k_i)}{M(k_v - k_i) - K_v k_i} \right]^2 \quad \text{(10b)}
\end{align*} \]

Inequalities (eqns 10a and 10b) describe relationships between the maximal values of the rates $V_1$, $V_2$ and $V_3$, as:

$$V_{\text{max}} = k_i M^2, \quad V_{\text{max}} = k_i \frac{M}{K_2 + M}, \quad V_{\text{max}} \approx k_i \tag{11}$$

From the consideration that $K_v \approx M$ (see above), it follows that $V_{\text{max}} \approx k_i/2$, and the inequalities (eqns 10a and 10b) become:

$$V_{\text{max}} \approx V_{\text{max}} \tag{12a}$$

$$V_{\text{max}} > V_{\text{max}} \tag{12b}$$

where $a_2 = V_{\text{max}}/V_{\text{max}}$. Inequalities (eqns 10a, 10b, 12a and 12b) describe the relationships between the $V_{\text{max}}$ values required to obtain high steady-state $[\text{Gα}\text{GTP}]$. Violation of these inequalities results in low steady-state $[\text{Gα}\text{GTP}]$.

If the steady-state $[\text{Gα}\text{GTP}]$ is low (<$K_v$), $V_3$ is no longer constant, but can be rewritten in a linear form. We decided to simplify $V_3$ into the linear form as well, as in the steady-state situation of trimeric G-protein activation, the concentration of the undissociated trimeric G-protein complex, $\text{Gα}\text{GTP}\beta\gamma$, is low (<$M \approx K_v$). Thus $V_2$ and $V_3$ become:

$$V_2 = \frac{k_i n_1}{K_v}; \quad V_3 = \frac{k_i n_3}{K_v} \tag{13}$$

Rewriting eqns (6a) and (6b) and solving them for the steady-state gives the following solutions:

$$[\beta \gamma] = n_2 = \frac{M}{2(b_3 + 1)} \left[ b_2 - b_2 + \sqrt{(b_3 + b_3)^2 + 4b_2} \right]$$

$$[\text{Gα}\text{GTP}] = n_3 = b_2(M - n_2) = b_2M \left\{ \frac{1}{2(b_3 + 1)} + \frac{1}{2(b_3 + 1)} \right\}$$

where $b_i \equiv W_i / W_j$, such that $W_i = k_i M^2 = V_{\text{max}}, \quad W_2 = k_2 M / K_v$, and $W_3 = k_3 M / K_v$. Here $W_2$ and $W_3$ are maximal rates for $V_2$ and $V_3$ written in the linear form (eqn 13). In eqn (14), solutions with a negative square root are discarded as being ‘non-biological’.

The steady-state analysis should be started with analysis of inequalities (eqns 12a and 12b). If they are fulfilled, the steady-state concentrations can be estimated using eqns (9a) and (9b). If not, eqn (14) should be used.

**Modelling feedback reactions in the trimeric G-protein cycle**

To model the positive feedback of $\text{Gα}\text{GTP}$-enhanced GPCR activity, the $V_2$ rate equation (eqn 3) can be rewritten in the following form, after [22]:

$$V_2 = k_2 \frac{n_1}{K_v + n_1} - \frac{1 + (B n_3 / K_\beta)}{1 + (n_3 / K_\beta)} \tag{15}$$

where $B$ and $K_\beta$ are kinetic constants. In Figure 7, $B$ is set as 100, and $K_\beta$ is set as 500 nM.

To model the negative feedback of $\text{Gα}\text{GTP}$-induced receptor internalization (removal from the cell surface), $[\text{Rc}]$ was set as a variable rather than as a constant:

$$\frac{d[Rc]}{dt} = V_{\text{del}} - V_{\text{rem}} = V_{\text{del}} - k_{\text{rem}}[\text{Rc}] \frac{1 + (A n_3 / K_\alpha)}{1 + (n_3 / K_\alpha)} \tag{16}$$

where $V_{\text{del}}$ is the rate of receptor delivery to the cell surface, $k_{\text{rem}}$ is the rate constant for receptor removal, and $A$ and $K_\alpha$ are kinetic constants (set as 0.3 nM$^{-1}$ s$^{-1}$, 0.01 s$^{-1}$, 180 and 400 nM respectively in Figure 7).

**Computer modelling**

Kinetic modelling was performed with the PLAS (Power Law Analysis and Simulation) software [19] (http://www.dgb.fc.ul.pt/docentes/aferreira/plas.html) using two numerical solvers: the
Figure 2 Four signalling modes predicted from kinetic modelling of the trimeric G-protein cycle

Receptor activation starts at zero time. Mode 1 is sustained production of high levels of $\beta\gamma$ and $G_{\text{GDP}}$, but not $G_{\text{GTP}}$, upon receptor activation. Mode 2 is production of high levels of $\beta\gamma$ and $G_{\text{GTP}}$. Mode 3 is a sustained production of high levels of $\beta\gamma$, with $G_{\text{GTP}}$ produced transiently and then falling to low levels, while mode 4 stabilizes $G_{\text{GTP}}$ at high levels after the original overshoot. The kinetic parameters in all four modes are: $k_{\text{diss}} = 0.001 \text{nM}^{-1} \cdot \text{s}^{-1}$ (modes 1, 3, and 4), and 0.01 $\text{nM}^{-1} \cdot \text{s}^{-1}$ (mode 2); $k_{\text{hydr}} = 15 \text{s}^{-1}$ (mode 1), 20 $\text{s}^{-1}$ (mode 2) and 25 $\text{s}^{-1}$ (modes 3 and 4); $k_{\text{act1}} = 1 \text{nM}$; $K_2 = 500 \text{nM}$; $K_3 = 2 \text{nM}$. Other parameters are: $k_{\text{M1}} = 30 \text{nM}$ (modes 1 and 2), 100 nM (mode 3) and 60 nM (mode 4); $\text{[GAP]} = 25 \text{nM}$ (modes 1 and 4), 20 nM (mode 2) and 52 nM (mode 3).

BDF stiff integrator for rate-law systems and Taylor series method for power-law systems.

RESULTS

Description of the four kinetic modes in the trimeric G-protein system

The kinetic model of the trimeric G-protein cycle (Figure 1) is described in the Materials and methods section. Kinetic modelling of this simple trimeric G-protein cycle yields surprising diversity in the signalling responses (Figure 2) in the range of parameters, quantified from experimental data (see the Materials and methods section). This diversity is largely manifest as the Go responses, represented as predominant production of $G_{\text{GTP}}$ over $G_{\text{GDP}}$, or vice versa; furthermore, these responses can be transient (Figure 2). In contrast, $\beta\gamma$ production is mostly sustained and uniform in response to receptor activation, and varies mostly in its level. We operationally define levels of components of the trimeric G-protein cycle ($G_{\text{GDP}}$, $G_{\text{GTP}}$, $\beta\gamma$, $G_{\beta\gamma}$) as 'high' if they exceed $K_1$ (the Michaelis–Menten constant for the GAP-activated GTP hydrolysis on $G_{\text{GTP}}$; set as 2 nM or < 1% of the total G-protein concentration) and 'low' if they do not.

The observed diversity in G-protein responses can then be categorized into four distinct signalling modes (Figure 2): sustainable production of high concentrations of $\beta\gamma$ and $G_{\text{GTP}}$ with low levels of $G_{\text{GDP}}$ (mode 1); sustainable production of high concentrations of $G_{\text{GTP}}$ and $\beta\gamma$ (mode 2); transient production of high concentrations of $G_{\text{GTP}}$ with sustained high $\beta\gamma$ (mode 3); and sustainable production of high concentrations of $G_{\text{GTP}}$ and $\beta\gamma$ with an initial overshoot in $G_{\text{GTP}}$ production (mode 4). This categorization has a biological rather than mathematical meaning: 'high' concentrations are implied to induce signal transduction, whereas 'low' are not. Furthermore, modes 3 and 4 are close to the mathematical description, but are vastly different biologically, as only in mode 4 can the stable activation of $G_{\text{GTP}}$-dependent responses be expected.

The conventional view of GPCR signalling implies that steady-state concentrations of $G_{\text{GTP}}$ and $\beta\gamma$ rise to high upon receptor stimulation. It is apparent from modelling presented here that this type of G-protein response is just one of multiple kinetic modes of G-protein activation (mode 2). The other three modes have not been previously characterized. The four modes of the trimeric G-protein cycle predicted here will probably produce a wider range of intracellular responses than just a sustainable production of $G_{\text{GTP}}$ plus $\beta\gamma$. Specifically, $G_{\text{GTP}}$-dependent cellular responses cannot be expected in mode 1, and can only be transient in mode 3. Numerical analysis shows that the duration of the $G_{\text{GTP}}$ transient in modes 3 and 4 is mostly under the negative control by the efficiency/concentration of the GAP, while the amplitude is largely determined by the efficiency/concentration of the activated receptor (Figure 3).

Steady-state analysis

The four kinetic modes can be placed in two separate groups: modes 2 and 4 showing high steady-state concentrations of both $\beta\gamma$ and $G_{\text{GTP}}$; and modes 1 and 3 showing high steady-state concentration of $\beta\gamma$ and low steady-state concentration of $G_{\text{GTP}}$. To assess whether the steady-state $[G_{\text{GTP}}]$ is high or low, inequalities (eqns 12a and 12b) should be used to compare the maximal levels of the $V_1$–$V_3$ rates. If $V_{2\text{max}} > V_{1\text{max}}$, inequalities (eqns 12a and 12b) should be used to compare the maximal levels of the $V_1$–$V_3$ rates. If $V_{2\text{max}} > V_{1\text{max}}$, $V_{1\text{max}} > V_{3\text{max}}$, $[G_{\text{GTP}}]$ will be high in the steady-state. In this situation, to calculate the steady-state concentrations of $[G_{\text{GTP}}]$ and $[\beta\gamma]$, eqns (9a) and (9b) should be used instead.

Prediction of the kinetic modes

Three rates determine the behaviour of the trimeric G-protein cycle: the rate of dissociation of the trimeric complex ($V_1$), the rate of GTP hydrolysis on $G_{\text{GTP}}$ ($V_2$), and the rate of re-association of the trimeric complex ($V_3$). The choice between the four signalling modes is determined by the maximal values of these three rates. Numerical analysis reveals that:

- mode 1 (low $G_{\text{GTP}}$ production) is achieved if $V_{1\text{max}} > V_{2\text{max}}$;
- mode 2 (sustained high $G_{\text{GTP}}$ production) is achieved if $V_{1\text{max}} > V_{2\text{max}} > V_{3\text{max}}$;
- mode 3 (transient $G_{\text{GTP}}$ production) is achieved if $V_{1\text{max}} > V_{2\text{max}} > V_{3\text{max}}$; and
- mode 4 ($G_{\text{GTP}}$ production with overshoot) is achieved if $V_{1\text{max}} > V_{2\text{max}} > V_{3\text{max}}$ (17)

where $V_{\text{max}}$ values are as defined in the Materials and methods section (eqn 11). Figure 4 illustrates the dependence of kinetic modes on the $V_{1\text{max}}$, $V_{2\text{max}}$ and $V_{3\text{max}}$ parameters.

Let us consider the situation when $V_{1\text{max}} > V_{2\text{max}}$. This situation is one of the two conditions implying low steady-state [$G_{\text{GTP}}$] (see inequalities in eqns 12a and 12b). As $V_3$ approaches $V_{3\text{max}}$ if $[G_{\text{GTP}}] > K_3$, the situation of $V_{1\text{max}} > V_{3\text{max}}$ also means that, for all noticeable concentrations of $G_{\text{GTP}}$, the rate of its destruction through hydrolysis will be higher than the rate of its production through dissociation of the $G_{\text{GTP}}$ $\beta\gamma$ trimer. Thus $[G_{\text{GTP}}]$ will always be low (mode 1), and $[\beta\gamma]$ will depend on $V_{3\text{max}}$. In essence, $[\beta\gamma]$ can be considerably below $M$ only in mode 1. All other modes require $V_{2\text{max}} > V_{3\text{max}}$, and have high $[\beta\gamma]$ close to $M$. 
Kinetic mode 3 describes the situation when \([G\alpha_{GTP}]_{\text{initial}}\) first rises to high but then falls back to low. Numerical and analytical calculations show that mode 3 occurs when the maximal rates relate as \(V_{2\text{max}} > V_{3\text{max}} > V_{1\text{max}}\). The condition \(V_{2\text{max}} > V_{3\text{max}}\) initially leads to fast dissociation of the \(G\alpha_{GDP}\) trimers, forming high concentrations of free \(\beta\gamma\) and \(G\alpha_{GTP}\). Later, the system adopts the steady-state in which the G-proteins will mostly exist in the form of \(\beta\gamma\) and \(G\alpha_{GDP}\). Indeed, the condition \(V_{3\text{max}} > V_{1\text{max}}\) is the other situation when the steady-state \([G\alpha_{GTP}]\) cannot be high (see inequalities in eqns 12a and 12b).

Similar analysis shows that mode 2 exists if \(V_{3\text{max}} > V_{1\text{max}} > V_{2\text{max}}\), and mode 4 exists if \(V_{2\text{max}} > V_{1\text{max}} > V_{3\text{max}}\).

Flips between modes

The multimodal behaviour of the trimeric G-protein cycle implies that the system can flip from one mode to another upon changes in concentrations of the components of the trimeric G-protein system. Such changes may arise from, e.g., increasing the concentration of the extracellular stimulus, reducing the plasma membrane levels of the receptor or changing the total receptor, GAP or G-protein levels. Figure 5 shows an example in which a system is predicted to flip from mode 1 (low \(G\alpha_{GTP}\) production) to mode 2 (sustained high \(G\alpha_{GTP}\) production) and back owing to changes in the levels of activated receptor and GAP. First, stimulation of a cell with low levels of a ligand yields relatively low concentrations of the activated receptor, which nevertheless elicits significant mode 1 responses (Figure 5). An additional increase in the ligand concentration stimulates the cell further, forcing the system to flip into mode 2. Cellular responses in these two modes would obviously be different. With time, a negative-feedback loop leading to enhanced production of the GAP protein can be activated; a 2.5-fold increase in the GAP concentration forces the system in Figure 5 back to mode 1, which would lead to termination of \(G\alpha_{GTP}\)-mediated responses. Importantly, changes in intracellular GAP concentration similar to those used in the modelling of Figure 5 have been observed experimentally upon prolonged stimulation of yeast cells with the mating hormone [10].

The steady-state flux

At the steady state, the three rates governing the G-protein system equalize. The resultant steady-state flux rate determines the velocity of turnover of the trimeric G-protein through the trimeric, active and monomeric inactive conformations. We have found that the steady-state flux rate is determined by the smallest of the \(V_{\text{max}}\) values in eqn (17). For example, mode 2 occurs if \(V_{1\text{max}} > V_{2\text{max}} > V_{3\text{max}}\) and the steady-state flux rate is determined by \(V_{3\text{max}}\), but is practically independent of \(V_{1\text{max}}\) or \(V_{2\text{max}}\). Figure 6 illustrates this feature. The steady-state flux rate is approximately linearly proportional to the lowest \(V_{\text{max}}\) value, while steady-state concentrations of \(\beta\gamma\) and \(G\alpha_{GTP}\) are affected to a considerably lower degree. For example, a 4-fold increase in GAP concentration (and thus \(V_{1\text{max}}\)) on Figure 6 leads to a 4-fold increase in the
The kinetic mode can be determined based on comparison of the maximal levels of the three rates.

Top left: mode 3 occurs if $V_{2\text{max}} > V_{3\text{max}} > V_{1\text{max}}$. Increase in $V_{1\text{max}}$, so that $V_{2\text{max}} > V_{1\text{max}} > V_{3\text{max}}$, leads to mode 4 (middle left). Further increase in $V_{1\text{max}}$, so that $V_{1\text{max}} > V_{2\text{max}} > V_{3\text{max}}$, leads to mode 2 (bottom left). Increase in $V_{3\text{max}}$, so that $V_{3\text{max}} > V_{2\text{max}}$, leads to mode 1, regardless of whether $V_{1\text{max}}$ is high (bottom right) or low (top right).

Figure 4

The trimeric G-protein signalling system can flip from one mode to another and back upon changes in conditions. At zero time, the system is stimulated with 1 nM activated GPCR, and later with 5 nM; both stimulations result in increasing activation of the system in mode 1. Further increase in the GPCR activation (20 nM and then 90 nM) results in a switch to mode 2 of system activation. Concentration of the GAP is originally modelled at 10 nM; stepwise increases in GAP concentration lead to reduction and then loss of the $G_{\alpha}\text{GTP}$ levels, switching the system back to mode 1; importantly, the system is still activated, and levels of $\beta\gamma$ remain high. Kinetic parameters, other than those shown, are as in Figure 3, except for $k_{\text{ass}}$, which is $0.0005 \text{nM}^{-1} \cdot \text{s}^{-1}$.

Figure 5

The trimeric G-protein signalling system can flip from one mode to another and back upon changes in conditions. At zero time, the system is stimulated with 1 nM activated GPCR, and later with 5 nM; both stimulations result in increasing activation of the system in mode 1. Further increase in the GPCR activation (20 nM and then 90 nM) results in a switch to mode 2 of system activation. Concentration of the GAP is originally modelled at 10 nM; stepwise increases in GAP concentration lead to reduction and then loss of the $G_{\alpha}\text{GTP}$ levels, switching the system back to mode 1; importantly, the system is still activated, and levels of $\beta\gamma$ remain high. Kinetic parameters, other than those shown, are as in Figure 3, except for $k_{\text{ass}}$, which is $0.0005 \text{nM}^{-1} \cdot \text{s}^{-1}$.

Oscillations

It is well established that GPCR-elicited signalling over time stimulates uncoupling of the GPCR from G-proteins, either through the action of protein kinases A or C, or through the action of GRKs (GPCR kinases) and $\beta$-arrestins [24]. The GRK/$\beta$-arrestin
signalling can lead further to receptor internalization. This regulation serves as a negative feedback terminating the response, with uncoupling being a faster acting, and internalization being a slower-acting negative feedback. Kinetic modelling confirms response termination by the negative feedback (Figures 7A and 7B). Similar modelling results are obtained for $\beta\gamma$- or G$\alpha$GTP-initiated negative feedback on GPCRs, and both can operate in cells [25].

Existence of the negative-feedback loop in the trimeric G-protein cycle makes the system highly sensitive to incorporation of additional feedback regulation. For example, experiments and modelling show that coexistence of a positive- and a negative-feedback loop can result in oscillations in concentrations of system components [22,26,27]. In agreement with this, we show here that addition of a positive feedback to the trimeric G-protein cycle with signal-mediated receptor uncoupling or internalization leads not to termination of the response, but to stable oscillations in the trimeric G-protein cycle activation in a broad range of conditions (Figures 7D and 7E). The nature of the positive-feedback loop shown in Figure 7 is G$\alpha$GTP-mediated activation of the GPCR-catalysed dissociation of the trimeric G$\alpha$GDP$\beta\gamma$ complex. Similar results are obtained by the $\beta\gamma$-mediated positive-feedback loop. Such stimulation of the GPCR by G$\alpha$GTP or $\beta\gamma$ can be either direct or indirect. The frequency and shape of the predicted oscillations in the trimeric G-protein cycle depend on the initial conditions (Figures 7D and 7E), such as the enzymatic potency of the receptor, or the nature of the GAP protein utilized.

Possible implications of the described variability in trimeric G-protein responses are elaborated in the Discussion.

**DISCUSSION**

The main result of the present study is a prediction of an unappreciated diversity in kinetic and steady-state responses of the trimeric G-protein system upon activation. Specifically, four distinct kinetic modes of the system behaviour are predicted: sustained production of $\beta\gamma$ and G$\alpha$GDP with only low levels of G$\alpha$GTP (mode 1); sustained production of both $\beta\gamma$ and G$\alpha$GTP (mode 2); transient production of G$\alpha$GTP with sustained $\beta\gamma$ (mode 3); and sustained production of both $\beta\gamma$ and G$\alpha$GTP with an initial overshoot in G$\alpha$GTP production (mode 4) (Figure 2). We performed extensive analysis of the experimentally measured parameters, such as kinetic constants and concentrations of components (see the Materials and methods section). The four kinetic modes exist in the identified range of parameters, and the choice between the four modes depends on the exact combination of parameter values. Once activated, the system can flip from one mode to another if parameters change (Figure 5). The prediction of choice of the kinetic mode by the G-protein system can be made by comparing the maximal values the three rates governing the trimeric G-protein cycle (eqn 17), these rates being the rate of formation of the trimeric G-protein complex ($V_1$), the rate of GPCR-catalysed dissociation of the complex ($V_2$) and the rate of GAP-accelerated hydrolysis of GTP on G$\alpha$-subunits ($V_3$).
Figure 7  Feedback loops can produce oscillations in the trimeric G-protein cycle

Mode 2 trimeric G-protein signalling (A) becomes transient upon incorporation of the negative-feedback loop of $G_{\alpha GTP}$-stimulated receptor internalization (B). Further addition of the positive-feedback loop of $G_{\alpha GTP}$-mediated stimulation of receptor activity induces oscillations in the trimeric G-protein cycle (D, E). The frequency and shape of oscillations depend on properties of the system: lower receptor/GAP activity ratio produces lower frequency (D) than does the higher receptor/GAP activity ratio (E). Strong off-balance activity of one enzymatic component prevents oscillations (C). Curve presentation is as in Figure 3. Kinetic parameters, other than in Figure 3, are: $k_{asc} = 0.01 \text{nM}^{-1} \cdot \text{s}^{-1}$; $k_{diss} = 20 \text{s}^{-1}$ (in A–C and E) and 18 s$^{-1}$ (in D); $k_{hyl} = 3 \text{s}^{-1}$ (in A–C and E) and 6 s$^{-1}$ (in D); $[Rc^*] = 50 \text{nM}$ (in A–C and E) and 20 nM (in D); $[\text{GAP}] = 40 \text{nM}$ (in A, B, D and E) and 100 nM (in C). Time scale is 15 s in (A) and (B), and 300 s in (C–E).
The typically assumed way of trimeric G-protein activation is production of high steady-state concentrations of both $\beta\gamma$ and GoGTP in response to cell stimulation – mode 2 in our classification. Although several models for GPCR–trimeric G-protein activation have been proposed previously [7–11,28], the three other kinetic modes that we describe here have not been predicted before. Our kinetic modelling shows that mode 2 can only be achieved if among the three rates governing the G-protein cycle, $V_{\text{max}}$ is the highest, and $V_{\text{max}}$ is the lowest (eqn 17). Previous models strongly overestimate $V_1$ and/or strongly underestimate $V_3$, thus being biased towards mode 2 and omitting the other modes. Several models are built implying $V_1$ to be not rate-limiting and the reaction of re-association of the trimeric G complex from $\beta\gamma$ and the reaction of GAP proteins, thus underestimating $V_2$. As shown in the Materials and methods section, the kinetic modes 3 and 4, which require that $V_{\text{max}}$ is not the highest of the three rates, thus could not be predicted by the previous models. 

Some earlier models were built without any consideration of the role of GAP proteins, thus underestimating $V_{\text{max}}$ by orders of magnitude [11,28]. In other models [7,9], the $k_{\text{hydro}}$ constant for the Sst2p (yeast GAP)-catalysed GTP hydrolysis on Gpa1p (yeast Go) was set at 0.11 s$^{-1}$ which rather corresponds to the basic GAP-independent GTP hydrolysis rate for mammalian and also yeast Go subunits (0.05 s$^{-1}$ [3] and 0.21 s$^{-1}$ [10]) respectively. Such underestimations of $V_{\text{max}}$ make the previous models unable to predict the kinetic mode 1, which requires that $V_{\text{max}} > V_{\text{max}}$ (eqn 17).

The form of equations used to model the GPCR-catalysed dissociation of the trimeric complex ($V_2$) and the GAP-catalysed hydrolysis of GTP ($V_3$) are usually not of the Michaelis–Menten form in previous models. For example, both reactions are modelled as linear in [7,9]. The Michaelis–Menten reactions $V_2$ and $V_3$ can only be reduced to the linear form if the concentration of the substrate is much lower than the respective Michaelis–Menten constant. As shown in the Materials and methods section, $V_2$ can be reduced to the linear form only for the kinetic modes 1 and 3. In contrast, in kinetic modes 2 and 4 with high steady-state GoGTP, $V_3$ is not linear but constant.

Thus the main difference between our kinetic model and those published previously is a more careful assignment and description of the rates governing the trimeric G-protein cycle, which allowed us observe the unexpected diversity in kinetic responses of this system.

Current experimental techniques used to monitor trimeric G-protein activation in vitro or in cell populations can hardly detect the kinetic diversity in G-protein responses predicted here. First, these techniques analyse activation of the G-protein system normalized for a population of cells. As a result, the kinetic aspects of individual cell behaviour, such as transient GoGTP production or oscillations in the G-protein cycle, become overlooked. Secondly, current methods do not measure the concentrations of the components of the trimeric G-protein cycle, but rather the steady-state flux rates of the system. For example, activation of GTP hydrolysis on trimeric G-proteins upon cell stimulation is often taken as a sign of formation of high concentrations of free GoGTP and thus activation of GoGTP-dependent responses. However, such accelerated GTP hydrolysis shows only that the G-protein system is active and constantly goes through the trimeric G-protein cycle, while the concentration of GoGTP can in fact be minuscule (as in kinetic modes 1 and 3). Other techniques, focusing on concentrations of individual components, should be developed to monitor kinetic behaviour of the trimeric G-protein system in individual cells.

As current techniques measure the steady-state flux rates, rather than concentrations of the components of the G-protein system, some unexpected experimental observations become apparent. For example, under some conditions, increase in the concentration of the GAP proteins enhances not only the rate of GTP hydrolysis in GPCR-stimulated cell membranes ($V_3$), but also the rate of GPCR-induced incorporation of GTP into trimeric G-proteins ($V_2$) [8]. As our modelling shows (see the Results section and Figure 6), in kinetic modes 2 and 4, the steady-state flux rate is directly proportional to $V_{\text{max}}$. Thus an increase in the concentration of GAP, increasing $V_{\text{max}}$, will increase the steady-state flux rate and, as a result, all three rates governing the cycle, including the rate of GTP incorporation ($V_2$).

Analysis of the steady-state flux rates offers explanations for the paradoxical experimental observations that certain GPCR-triggered cellular responses are enhanced by addition of a GAP [29,30]. We propose that certain cellular responses are under the influence of the flux rate of G-protein turnover through the trimeric G-protein cycle, rather than solely on the absolute concentrations of active components of the trimeric G-protein system. A similar hypothesis was put forward for the small GTPase Rab5-controlled vesicular trafficking, where it was not the concentrations of the GTP- compared with GDP-bound forms of Rab5 that activated trafficking, but rather the speed of Rab5 cycling between the GTP- and GDP-bound forms [31]. In the case of trimeric G-proteins, when kinetic modes 2 or 4 are in place, increasing [GAP] will increase the flux rate and thus stimulation of some intracellular read-out mechanisms.

The four modes of the trimeric G-protein cycle predicted here will produce a wider range of intracellular responses than a sustainable production of GoGTP plus $\beta\gamma$. For example, mode 1 (production of high $\beta\gamma$ with low GoGTP) will elicit $\beta\gamma$-dependent, but not GoGTP-dependent, responses, and might be used in $\beta\gamma$-activated signalling in yeast mating [32] or leucocyte chemotaxis [33]. Mode 1 might also be involved in responses controlled by free GoGTP (e.g. in the GPCR-mediated control of asymmetric cell divisions [34]). Modes 3 and 4 (transient production of GoGTP) are attractive for explaining transient cellular responses to stimuli. So far, negative-feedback loops have been implicated in explaining such transient responses. In the present study, we show that such transience may be a result of intrinsic properties of the trimeric G-protein cycle.

Kinetic modelling shows that the duration of the GoGTP transient in modes 3 and 4 is determined largely by the efficiency/concentration of the GAP, while the amplitude is mostly determined by the efficiency/concentration of the activated receptor (Figure 3). As different signalling complexes comprising the activated receptor, trimeric G-protein complex and the GAP protein can emerge [5,35,36], they may result in production of different GoGTP transients decoded differently by the downstream signalling components.

The four kinetic modes arise in the experimentally measured kinetic and concentration range of parameters. For example, $k_{\text{on}}$ values for different Go subunits vary by one or two orders of magnitude [17], which leads to a prediction that trimeric G-proteins with higher $k_{\text{on}}$ (such as Gs) can be found with more likelihood in mode 2 than trimeric G-proteins with lower $k_{\text{on}}$ (such as Go, Gs) for which modes 3 and 4 are more likely.

GRK- and $\beta$-arrestin-dependent desensitization/internalization of GPCRs after prolonged stimulation is a well-known feedback mechanism of adaptation and signal termination [24]. Interestingly, a body of evidence shows that the GPCR desensitization pathway is also required for obtaining certain cellular
responses and not just termination of cell activation. For example, leucocyte chemotaxis is defective in mice lacking GRK6 or β-arrestin 2 [37]. Chemotactic leucocytes exhibit short- and long-scale oscillatory behaviour (with periods of approx. 10 and 45 s) necessary for chemotaxis [38]. One might speculate that repetitive termination of the GPCR response, mediated by the GRK6/β-arrestin 2, directs this oscillatory chemotactic behaviour. Similarly, some GPCR-stimulated oscillations in intracellular Ca2+ are dependent on protein kinase C-mediated GPCR uncoupling [39,40], supporting the hypothesis that cyclic GPCR activation translates into Ca2+ oscillations [41].

However, kinetic modelling of the G-protein cycle with the negative feedback does not produce oscillations, but a single transient response (Figure 7B). [Under some marginal conditions, damped oscillations can be obtained (results not shown).] However, addition of a positive feedback to such modified G-protein system produces oscillations under a broad range of conditions.

The nature of the positive-feedback loop in our modelling is GoGTP – or βγ-mediated activation of the GPCR-catalysed dissociation of the trimeric GoGDPβγ complex. Such stimulation of the GPCR by GoGTP or βγ can be either direct or indirect, and may include enhancement of the enzymatic activity of the receptor or increase in the affinity of ligand–receptor interaction. Potentially, the positive feedback could be achieved by substrate inhibition of the GAP-catalysed GTPase reaction [22]. Free βγ has been found to inhibit the GAP-catalysed GTPase reaction [5,42]. However, the mechanism of this inhibition appears to be competitive, with βγ increasing the apparent Michaelis–Menten constant of the reaction, without changing Vmax [43]. Incorporation of such inhibition into the kinetic modelling did not significantly change the behaviour of the system and was not sufficient to induce oscillations (results not shown).

The frequency and shape of the predicted oscillations in the trimeric G-protein cycle depend on the initial conditions (Figures 7D and 7E), such as the properties of the receptor and the GAP. They also depend on other receptor properties, such as dephosphorylation/trafficking rates. Thus a plethora of kinetic responses can be elicited within a cell by activation with different stimuli, even if the same or similar G-protein-complexes are used to transduce them. The downstream signalling components are then predicted to read different oscillations of the G-protein cycle differently, which would then elicit different cellular responses.

Several experimental observations are explained by the kinetic modelling of oscillations presented here. For example, it has been shown that a large increase in GAP concentration turns GPCR-induced Ca2+ oscillations into a sustained Ca2+ increase [44]. This is in a good agreement with the cessation of trimeric G-protein oscillations predicted to result from a large increase in GAP concentration (Figure 7C).

Experimental demonstration of the positive feedback in the trimeric G-protein system is lacking so far, but is a frequent phenomenon in a variety of chemical, metabolic, genetic or signalling cascades (see, e.g., [27,45,46]). Such a positive feedback is known to exist in the endocytotic traffic controlled by the small G-protein Rab5. There, membrane-bound GTP-loaded Rab5 molecules recruit a complex of the Rab5 effector Rabaptin-5 and the Rab5-specific GEF Rabex-5, thus amplifying the activation of membrane-bound Rab5 molecules [47]. We predict the existence of a similar positive-feedback loop in the trimeric G-protein cycle. Oscillation in the G-protein cycle would be able to convey a wealth of information (encoded in the form of oscillation frequency, shape and amplitude) to the downstream signalling components. Such G-protein oscillations can translate into oscillations in the activation of downstream signalling intermediates, demonstrated for cAMP [48], inositol 1,4,5-trisphosphate [40] and Ca2+ [39]. They may also be decoded by non-oscillating read-outs, perhaps similarly to the way different calcium oscillations translate differently into transcriptional responses [49].

The simple kinetic modelling shown in the present paper predicts an unappreciated diversity in G-protein responses, highlighting that behaviour of even simple systems can be far more complicated than intuitively expected. The diversity of G-protein responses comes in the format of four different kinetic modes and flips between them, as well as two different steady-state conditions (with high or low [GoGTP]). Furthermore, steady states with different flux rates can be produced. The model further serves as a framework to incorporate positive- and negative-feedback loops, predicting oscillations in the trimeric G-protein cycle. The following features (and their various combinations) of the trimeric G-protein cycle are predicted to determine specific activation of the downstream signalling: (i) the concentration of free βγ; (ii) the steady-state concentration of GoGTP; (iii) the amplitude and duration of the GoGTP transient; (iv) the steady-state flux rate; and (v) the oscillatory mode, the frequency and the amplitude of the βγ and GoGTP peaks.

This wealth of kinetic and steady-state responses produced by the simple trimeric G-protein system may serve to encode signal specificity in GPCR signalling.

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