Gₐ Activation Is Time-limiting in Initiating Receptor-mediated Signaling*

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To analyze individual steps of Gₛ-linked signaling in intact cells, we used fluorescence resonance energy transfer (FRET)-based assays for receptor-G protein interaction, G protein activation, and cAMP effector activation. To do so, we developed a FRET-based sensor to directly monitor Gₛ activation in living cells. This was done by coexpressing a Gₛ mutant, in which a yellow fluorescent protein was inserted, together with cyan fluorescent protein and appropriate receptors in HEK293 cells. Together with assays for receptor activation and receptor-G protein interaction, it is possible to characterize large parts of the GS signaling cascade. When A₂ₐ adenosine or β₁-adrenergic receptors are coexpressed with Gₛ in HEK293T cells, the receptor-Gₛ interaction was on the same time scale as A₂ₐ receptor activation with a time constant of <50 ms. Gₛ activation was markedly slower and around 450 ms with similar kinetics following activation of A₂ₐ or β₁ receptors. Taken together, our kinetic measurements demonstrate that the rate of Gₛ activation limits initiation of Gₛ-coupled receptor signaling.

Cellular communication is a major necessity in multicellular organisms. Responses to such diverse stimuli as light, odors, and hormones are mediated via G protein-coupled receptors (GPCRs) and their downstream signaling pathways (1, 2). Agonist-activated GPCRs activate in turn heterotrimeric G proteins, which are grouped according to their α subunits in four families. Among other effects, members of the Gₛ family stimulate the adenyl cyclase (AC), those of the G½ family inhibit it, activation of G₁α family G proteins leads to activation of phospholipase Cβ, and G₁₂/₁₃ family members activate small monomeric G proteins (3). However, the interplay of receptors, G proteins, and effectors is largely not understood. This especially holds true for kinetic aspects of such signaling cascades, with the exception of the rhodopsin/transducin system (4).

Apart from that, kinetic measurements in living cells have been possible for effectors of Gₛ family G proteins only because these G proteins directly activate G protein-activated inwardly rectifying K⁺ channels (5), and current flow through these channels can be assessed using the patch clamp technique. Unfortunately, this approach cannot be used to analyze signaling systems other than those involving Gₛ and to analyze effector activation and activity other than ion channels. Measurements of receptor activity, G protein activity, or cAMP levels mostly require cell lysates, which makes it hard to project the results to the situation in living cells.

In recent years, fluorescence resonance energy transfer (FRET) using genetically encoded fluorophores (6) opened the way to analyze protein-protein interactions directly in living cells (7). Specifically, this approach has been applied to analyze distinct steps in GPCR-mediated signal transduction, including receptor activation (8), receptor-G protein interaction (9), G protein activation (10–13), and measurements of the second messenger cAMP (14, 15). So far, direct measurement of G protein activation in mammalian cells has only been developed for G₁₅ proteins (10–12).

Therefore, we established a FRET-based assay to monitor Gₛ activation and deactivation kinetics in intact cells. Together with assays for other steps in the Gₛ signaling cascade, this enabled us to analyze the kinetics of large parts of the signaling cascade originating from these GPCRs. To directly compare kinetics for different steps, we used the same cellular system and similar expression levels of signaling molecules since these kinetics strongly depend on the amount of receptors expressed (16, 17). Specifically, we sought to answer the following questions. How are the time courses of signaling events related? Are there differences when the signal originates from different receptors? What can we learn by looking at deactivation processes? In the present study, we show that Gₛ activation limits the initiation of receptor-mediated signaling and that signaling kinetics are receptor-specific.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—The A₂ₐ-YFP adenosine receptor (A₂ₐ-R-YFP) was constructed by fusing YFP to the Smal site of the receptor cDNA, thereby truncating a part of the C terminus of the receptor; the β₁-YFP adrenergic receptor (Gly³⁸⁹; β₁-AR-YFP) was constructed by fusing YFP C-terminally to the cDNA using TCTAGA as linker. In radioligand binding and cAMP production, both constructs did not differ from their wild-type...
counterparts (data not shown). Ga\(_{\alpha}\)-YFP was constructed analogous to Ga\(_{\alpha}\)-CFP (18). CFP-\eG\(_{\gamma}\) and Epac1-camps have been described previously (14, 19).

Cell Culture—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% fetal calf serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin in an atmosphere of 95% air, 5% CO\(_2\). 0.5 units/ml penicillin in an atmosphere of 95% air, 5% CO\(_2\). 0.5 units/ml adenosine desaminase was added to the medium when A\(_{2A}\)-R or A\(_{2A}\)-R-YFP were cotransfected, and 100 \muM S-(−)-propranolol was added to β\(_{1}\)-AR and Epac1-camps-transfected cells. Cells were transfected either by using Effectene (Qiagen) according to the manufacturer’s instructions or by mixing of cDNAs for the indicated constructs with Dulbecco’s modified Eagle’s medium and 4.1 \mug of polyethyleneimine (Sigma) in a total volume of 214 \mu l; the mixture was incubated for 30 min and then put on cells. Amounts of DNA used per 3-cm dish were: A\(_{2A}\)-R-YFP, A\(_{2A}\)-R, β\(_{1}\)-AR-YFP, and β\(_{1}\)-AR, 0.4 \mu g; Ga\(_{\alpha}\), G\(_{\beta}\), and G\(_{\gamma}\), 2 \mu g; CFP-γ\(_{2}\) and Ga\(_{\alpha}\), 0.5 \mu g; and Epac1-camps, 0.5 \mu g. As judged by fluorescence, these transfection conditions led to more receptors than G protein subunits at the cell membrane. Special care was taken that examined cells had similar fluorescence levels. Cells were transfected 48 h prior to the experiments; for measurements of A\(_{2A}\)-R activation, HEK293 cells stably expressing A\(_{2A}\)-FLA4H/CFP-R were used.

**FRET Measurements and Imaging**—Cells were grown on poly-D-lysine-coated glass coverslips. Fluorescence microscopy was done with an Axiosvert 200 inverted microscope (Zeiss, Jena, Germany) using a ×63 oil immersion objective, a dual-photomultiplier system, and a polychrome IV (both from TILL Photonics, Gräfelfing, Germany). Illumination time was set to 436/153 ms at a frequency of 1–20 Hz. Excitation wavelength was set to 436 ± 10 nm (beam splitter dichroic long pass 460 nm), and emission of single whole cells was recorded at 535 ± 15 and 480 ± 20 nm (beam splitter dichroic long pass 505 nm). Special care was taken to ensure that fluorescence levels and distribution were similar in examined cells. FRET rates were calculated as ratios of YFP over CFP emission (F\(_{\text{YFP}}/F_{\text{CFP}}\), F\(_{\text{YFP}}\) was corrected for direct excitation (0.06 of F\(_{\text{YFP,total}}\) determined by direct excitation with 490 nm) and bleed-through (90% of F\(_{\text{CFP}}\)) (8–10). Measurements of CAMP levels were made as described (14), and confocal images were taken using a Leica TCS SP2 system (20).

Cells were continuously superfused with external buffer (in mM: 137 NaCl, 5.4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES at pH 7.3). For measurements involving β\(_{1}\)-AR, 200 nM ICI118,551 was added to the buffer to block endogenous β\(_{2}\)-ARs. Agonists were freshly prepared and applied using a rapid superfusion system allowing for solution exchange times of 5–10 ms (8).

**Data Processing**—Fluorescence intensities were acquired using CLAMPEX (Axon Instruments, Foster City, CA). Values are given as mean ± S.E. of \(n\) experiments, and statistical analyses and curve fitting were performed using Prism 4.0 (San Diego, CA) or CLAMPFIT (Axon Instruments).

**RESULTS**

Our aim was to better understand GPCR-linked signal transduction by analyzing the kinetics of different steps of the signaling cascade in a comparable cellular system. These steps included the interaction of the activated receptor with the G protein, G protein activation, and cAMP effector activation assessed as cAMP binding to the cAMP binding site of Epac1. We employed FRET-based assays for each of these steps, monitored single, intact cells, and chose A\(_{2A}\)-Rs and β\(_{1}\)-ARs as the origin of the signaling cascade.

First, we examined receptor-G protein coupling by measuring FRET between the YFP-tagged receptor and G\(_{\alpha}\) proteins, whose \(\gamma\) subunits were labeled with CFP (Fig. 1A). It has previously been shown that labeled receptors and G proteins remain functional (9–11). Confocal images of A\(_{2A}\)-R-YFP and Ga\(_{\alpha}\), G\(_{\beta}\), CFP-γ\(_{2}\) cotransfected cells demonstrated that the fluorescent constructs were located at the cell membrane (Fig. 1B) but also somewhat at intracellular membranes. Using bimolecular fluorescence complementation, it has been shown previously that the signal at the plasma membrane originates from G\(_{\beta}\), CFP-γ\(_{2}\) complexes, whereas the intracellular CFP fluorescence mainly originates from \(\gamma\) subunits not associated with a \(\beta\) subunit (18).

Single-cell fluorescence was measured while the cell was superfused with buffer or agonists. Application of adenosine led to a decrease in FCFP and an increase in FYFP and thus to an increase in ratiometric FRET (Fig. 1C, left). After agonist application, a small overshoot of the signal was observed before the FRET signal reached a plateau phase. The signal was readily reversible on agonist washout (see also below), and the amplitude depended on agonist concentration (data not shown). To determine the kinetics of the receptor-G protein interaction, an agonist concentration of 1 mM was used. By choosing this concentration, it was ensured that diffusion of the agonist to receptors did not become time-limiting (8, 9). A monophasic exponential curve was fitted to the data, and a time constant \(\tau_{\text{on}}\) of 49.8 ± 5.5 ms obtained (\(n = 7\); Fig. 1C, center). After withdrawal of 100 \muM adenosine, receptor-G protein dissociation kinetics were determined by fitting monoeponential curves to the data of single experiments. We have shown previously that, in accordance with the law of mass action, the interaction off kinetics do not depend on the concentration of agonist applied before (9). For the termination of the interaction of A\(_{2A}\)-R with G\(_{\alpha}\), a \(\tau_{\text{off}}\) of 14.8 ± 1.6 s was measured (\(n = 13\); Fig. 1C, right).

Next, we examined the interaction of another G\(_{\alpha}\)-coupled receptor with the G protein. This was done by cotransfection of β\(_{1}\)-AR-YFP and Ga\(_{\alpha}\), G\(_{\beta}\), CFP-γ\(_{2}\) and recording of single cell fluorescence. Again, application of the agonist norepinephrine led to a decrease in FCFP and an increase in FYFP and thus to an increase in ratiometric FRET (Fig. 1D, left). Interaction kinetics were determined using the saturating concentration of 1 mM norepinephrine and had a \(\tau_{\text{on}}\) of 58.1 ± 7.5 ms (\(n = 8\); Fig. 1D, center). Finally, determination of off-kinetics after agonist withdrawal yielded a \(\tau_{\text{off}}\) of 8.4 ± 1.0 s (\(n = 9\); Fig. 1D, right).

The interaction of receptors with G proteins is a prerequisite for G protein activation, which is the next step in the signaling cascade. We therefore developed a FRET-based sensor to measure G\(_{\alpha}\) activation in mammalian cells. FRET was measured between YFP inserted in Ga\(_{\alpha}\) and CFP-γ\(_{2}\) after cotransfection with G\(_{\beta}\) and an appropriate receptor (A\(_{2A}\)-R or β\(_{1}\)-AR) in HEK293T cells (schematically depicted in Fig. 2A). Confocal pictures of cells transfected with A\(_{2A}\)-R and Ga\(_{\alpha}\)-YFP, β\(_{1}\)-AR, and Ga\(_{\alpha}\)-YFP and Ga\(_{\alpha}\)-G\(_{\beta}\) were constructed analogous to Ga\(_{\alpha}\)-CFP (18).
CFP-γ2 showed that fluorescent constructs were localized at the plasma membrane but also intracellularly (Fig. 2B). Here, the intracellular part of Gαγ2-YFP fluorescence is due to our relatively high amount of transfected cDNA as it has been shown that the amount of plasma membrane fluorescence of Gαγ1-CFP is inversely correlated with the amount of DNA transfected (21); for the distribution of CFP-H9253 fluorescence, see above. We performed experiments analogous to those described above. First, we examined A2A-R-evoked responses by measuring single-cell fluorescence of cells cotransfected with A2A-R and Gαγ1-CFP-γ2. Application of 100 μM adenosine led to an increase in FCFP, a decrease in FYFP, and thus to a decrease in ratiometric FRET (Fig. 2C, left). After agonist washout, fluorescence and FRET ratio slowly recovered to initial levels. Gs activation kinetics were analyzed by fitting monoexponential curves to the exponential phase of the FRET trace. Thereby, a τon for Gs activation of 49.6 ± 5.5 ms was determined (n = 7; Fig. 2C, center). We examined the deactivation kinetics after washout of 100 μM adenosine by fitting a monoexponential curve, yielding a time constant of 14.8 ± 1.6 ms (n = 5; Fig. 2C, right). Again, these data were compared with those obtained when βγ1-ARs were stimulated initially. Here, similar to the results described for A2A-R-mediated Gs activation, stimulation of transfected cells with 100 μM norepinephrine...
decreased the ratiometric FRET signal (Fig. 2D, left). Fitting of monoeXponential curves revealed a \( \tau_{on} \) of 437 ± 54 ms for 100 μM norepinephrine-evoked G protein activation (via \( \beta_1 \))—ARs (\( n = 7; \) Fig. 2D, center), which was not significantly different from that of \( \alpha_{2A} \)-AR-evoked G protein activation (\( p = 0.38, \) two-tailed t test). Finally, \( G_\alpha \) \( \tau_{off} \) was determined as 15.0 ± 3.4 s following washout of 100 μM norepinephrine (\( n = 6; \) Fig. 2D, right).

Activated \( G_\alpha \) proteins activate the AC, thereby producing cAMP. We have previously developed a FRET-based sensor based on the cAMP binding domain of the exchange protein directly activated by cAMP (Epac1-camps) (14), which reports binding of cAMP as a decrease in FRET. Here, Epac1-camps was used to assess cAMP effector activation. Since changes in FRET of this sensor did not follow a monoeXponential decay, traces were analyzed by measuring the activation half-time, i.e. the time from agonist application until the signal reached 50% of its maximal amplitude. We again compared responses mediated via \( \alpha_{2A} \)-R and \( \beta_1 \)-AR by cotransfecting Epac1-camps and the respective receptor. Fig. 3A shows averaged FRET responses after stimulation of \( \alpha_{2A} \)-R and Epac1-camps-cotransfected cells with 100 μM adenosine, and an activation half-time of 30.7 ± 2.1 s was determined (\( n = 6 \)). In Fig. 3A, the inset shows a magnification of the first phase after initial stimulation demonstrating that the signal changes about 1 s after agonist application. However, when analyzing these experiments, it should be kept in mind that Epac1-camps is distributed within the whole cell and not only at the plasma membrane. It is reasonable to assume that effectors located near ACs get activated earlier than those further apart, an issue that cannot be addressed with FRET sensors presently available. Similar
results were obtained when cAMP production mediated by \( \beta_1 \)-ARs was analyzed; here, after stimulation with 100 \( \mu M \) norepinephrine, the activation half-time was 37.5 ± 6.8 s (Fig. 3B). Because we had determined a concentration-response curve for Epac1-camps previously (14), it is possible to calculate cAMP concentrations from cAMP-induced changes in FRET. The maximal change in FRET reported by Epac1-camps is 30%, corresponding to cAMP concentrations between 0.1 and 10 \( \mu M \). Since we also observe a 30% change after \( A_{2A} \)-R stimulation (Fig. 3A), it is fair to assume that, under our experimental conditions, cAMP concentrations in the cell before agonist stimulations are below 0.1 \( \mu M \) and reach at least 10 \( \mu M \) after agonist stimulations. However, given the sigmoidal relation of the Epac1-camps FRET signal and cAMP concentrations, the most sensitive range of the sensor is around cAMP concentrations of 1 \( \mu M \). Fig. 3C presents cAMP concentrations over time after stimulation of \( A_{2A} \)-R calculated from the FRET change as reported by Epac1-camps, with the inset showing the initial changes of calculated cAMP concentration.

Lastly, we sought to compare the data we obtained from measuring receptor-G protein interaction, G protein activation, and cAMP production. To directly compare the kinetics of these different steps, the data already obtained were supplemented with measurements of \( A_{2A} \)-R activation using the \( A_{2A} \)-FlAsH/CFP-R activation probe (22). Measurements of \( A_{2A} \)-R activation yielded a \( \tau_{on} \) of 37.7 ± 2.8 ms (\( n = 8 \)). Representative experiments for receptor activation, receptor-Gs protein interaction, and Gs activation were graphed in the same diagram (Fig. 4A). In Fig. 4B, time constants of experiments described above are collected in one graph; the \( \tau_{on} \) for \( A_{2A} \)-R activation was 37.7 ms; the \( \tau_{on} \) for \( A_{2A} \)-R-Gs interaction was 49.6 ms; and the \( \tau_{on} \) for Gs activation was 493 ms (see above). Finally, representative examples of \( A_{2A} \)-R deactivation (determined to have a \( \tau_{off} \) of 2.07 ± 0.5 s (\( n = 9 \)), data not shown), deactivation of \( A_{2A} \)-R-Gs interaction, and Gs deactivation are given in Fig. 4C.

FIGURE 3. cAMP effector measurements. A, averaged ratiometric FRET traces (black line; S.E. in gray) of HEK293T cells expressing \( A_{2A} \)-R and Epac1-camps after stimulation with 100 \( \mu M \) adenosine (bar). Activation half-time was determined as 30.7 ± 2.1 s (\( n = 6 \)). The inset magnifies the time course from 2 s before agonist application until 5 s after. B, averaged ratiometric FRET traces (black line; S.E. in gray) of cells expressing \( \beta_1 \)-AR and Epac1-camps after stimulation with 100 \( \mu M \) norepinephrine (bar) (activation half-time of 37.5 ± 6.8 s, \( n = 7 \)). C, changes in the concentration in intracellular cAMP (black line; S.E. in gray) calculated from FRET changes of Epac1-camps after stimulation of \( A_{2A} \)-AR with 100 \( \mu M \) adenosine (bar). A magnification of the initial phase is shown in the inset.

DISCUSSION

Traditional methods to assess G protein function mainly rely on cell-destructive methods. In recent years, FRET techniques have been used to study signaling processes in intact cells and in real time, which allowed analysis of signaling steps in a physiological setting (7). Since FRET assays require the proteins of interest to be labeled with fluorophores, it is crucial to make sure that they behave like their wild-type counterparts. In radioligand binding and cAMP production, the fluorescently tagged receptors used in this study (\( A_{2A} \)-R and \( \beta_1 \)-AR) do not differ from the respective wild-type receptors; \( A_{2A} \)-FlAsH/CFP-R has been described previously (22). Likewise, insertion of a GFP variant in Gs does not interfere with its signaling properties (18), as is the case for CFP-tagged G\( \beta \gamma \) subunits (10). This enabled us to set up an assay to directly monitor Gs activation in living mammalian cells, which has possible been previously only for G13\( \alpha \) proteins (10–13). Upon agonist stimulation of the receptor, FRET between labeled Gs and G\( \beta \gamma \) subunits of Gs decreases. Since this is caused by an increase of the distance between YFP and CFP, which in turn are fused to different Gs subunits, the distance between \( \alpha \) and \( \beta \gamma \) should also increase. The fact that a complete loss of FRET is not observed after agonist stimulation of receptors may be due to Gs and G\( \beta \gamma \) not dissociating completely; alternatively, this may reflect the steady state of the G protein cycle. As judged by fluorescence,
examinecd cells had more receptors than G proteins at the cell membrane. Therefore, it is unlikely that functional G proteins remain inactive because of a lack of receptors available for their activation.

G protein activation is a time-limiting step in this signaling pathway. The interaction of the A2A-R with G protein can be in the time frame of A2A-R activation (~40 ms) and thus not be time-limiting in the context of high expression of signaling molecules. This may reflect certain physiological settings that do include high receptor and G protein densities such as post-synaptic membranes. We have demonstrated previously that the interaction of the A2A-R adrenergic receptor and G protein becomes time-limiting when only endogenous G proteins are present; this leads to a roughly 2-fold increase in the time constant from 44 to 86 ms (9). Moreover, our results are in line with a recent bioluminescence resonance energy transfer study investigating \( \beta_{2A} \)-AR-G protein interaction (23). That study reported interaction half-times of around 300 ms, and the slower activation time constant reported there may be due to the agonist application via an injection system. However, when comparing receptor-G protein interaction with G protein activation, we found that A2A-R-evoked G protein activation is around 10-fold slower than receptor activation and the initial interaction. Although receptor-G protein interaction is already maximal after 50 ms, at this time point, only a fraction of G protein is activated, and it takes several hundred milliseconds until G proteins are maximally activated. The activation time constant of G protein is similar to that of G proteins (10).

The difference in time courses of receptor-G protein interaction and G protein activation is similar to the difference observed with the \( \alpha_{2A} \)-adrenergic receptor and G proteins (8–10). There are at least three events between receptor-G protein interaction and G protein activation, namely GDP release, GTP binding, and at least one conformational change, and it is believed that GDP release from the G protein is rate-limiting in G protein activation (24, 25).

cAMP effector activation was assessed using Epac1-camps, which is based on the cAMP binding site of Epac (14). Half-times of signal change of this sensor were ~35 s and thereby much slower than the initial steps of the G protein signaling cascade. However, the first change of the signal occurred more rapidly, i.e. in the order of a few seconds after agonist application. A recent publication by Rebois et al. (26) showed that G protein and ACs are complexed regardless of whether or not signal transduction was initiated by receptor agonists; similarly, there is evidence that G protein is stably associated with its effector phospholipase C Beta 1 (27). The association of G proteins and effector suggests that the latter is activated quickly. Nevertheless, it is obvious that changes in whole-cell concentration of a second messenger, e.g. cAMP, need substantially longer to be established when compared with initial signaling steps. Future studies with direct measurements of effector activation are needed to analyze these processes.

As initial transducers of the G protein signaling cascade, we used adenosine-stimulated A2A-R and norepinephrine-stimulated \( \beta_{1} \)-R. The interaction kinetics of the A2A-R were not significantly different from those of the \( \beta_{1} \)-AR with G protein. Of these two receptors, kinetic data are currently only available for the A2A-R (22): these were confirmed in this study to be ~40 ms. Thus, at the A2A-R, receptor activation and subsequent receptor-G protein interaction can be on the same time scale. Given the similar kinetics for receptor-G protein interaction, we consequently do not observe a major difference in G protein activation when comparing activation via A2A-R and \( \beta_{1} \)-AR; here, both time constants were markedly slower (494 ± 31 and 437 ± 54 ms for A2A-R and \( \beta_{1} \)-AR, respectively), indicating that G protein activation is limited by a different step from the interaction. This also implies that steps downstream of G protein activation should occur on a similar time scale, and this notion is supported by the fact that the speed of receptor-stimulated cAMP accumulation is very similar (half-times of ~35 s for A2A-R and \( \beta_{1} \)-AR-mediated signals).

Although the activation processes mediated via A2A-R and \( \beta_{1} \)-AR look similar, major differences were observed when deactivation kinetics were analyzed. Here, both termination of A2A-R-G protein activation and deactivation of A2A-R-activated cAMP
proteins were considerably slower than the respective steps involving βγ-ARs. Hypothetical reasons for the slower termina-
tion of the interaction are a slower washout of the more
lipophilic agonist adenosine or different agonist off-kinetics
from the receptors. However, both are unlikely due to the fact
that A2A-R deactivation had a time constant of ∼2 s. Where
does this difference then come from? First, the receptor deac-
tivation represents agonist wash-out from receptors, whereas
the termination of the receptor-G protein interaction corre-
sponds to the termination of the ternary complex of agonist,
receptor, and G protein. Moreover, the agonist may be retained
by some high affinity receptors (i.e. receptor-G protein com-
plexes), which is not seen when receptor deactivation is ana-
lyzed (since the majority of receptors are not in a high affinity
state). The situation at A2A receptors is similar when compared
with α2A-adrenergic receptors; at the latter, receptor deactiva-
tion also had a time constant of about 2 s, whereas the termina-
tion of receptor-Gi interaction had a τoff of ∼13 s (8, 22). Con-
cerning the overshoot of the FRET signal seen with A2A-R-Gi
interaction, this can also be explained by the slower off-kinetics.
Immediately after agonist stimulation, the FRET signal is
increased by the association of receptors with G proteins,
whereas it takes some time until the dissociation starts and a
steady state is reached. This view is supported by the notion
that this overshoot is not seen readily with the
steady state is reached. This view is supported by the notion
that this overshoot is not seen readily with the
steady state earlier.

We demonstrated that the termination of receptor-G protein
interaction is faster than G protein deactivation after A2A-R
activation. The fact that the deactivation of receptor-G protein
interaction represents the dissociation of the ternary complex
of agonist, receptor, and G protein demonstrates that agonist
washout is not limiting in this situation. Therefore, we specu-
late that the difference in Gs protein deactivation time con-
stants may more likely be caused by different activities of
members of the regulators of G protein signaling (RGS) protein
family, which can act as GTPase-accelerating factors (28). It has
been shown that RGS proteins can be associated with distinct
receptors (29, 30), and this could be the reason for the differ-
ence in G protein deactivation kinetics observed here. None-
theless, although Gs specific RGS proteins have been described
(31), so far, no receptor specificity of RGS proteins for Gs has
been reported. Thus, the difference in off-kinetics has to be
examined in subsequent studies in more detail.

Taken together, our analyses of different steps of Gs signaling
pathways in living cells demonstrate fast encounters of G pro-
teins with activated receptors. This is followed by G protein
activation, which represents a time-limiting step in signal
transduction. As a consequence of their activation, α and βγ
subunits of Gs dissociate or at least reorient, resulting in AC
activation by activated Go.

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