Many physiological and pathophysiological processes are regulated by cAMP. Different therapies directly or indirectly influence the cellular concentration of this second messenger. A wide variety of receptors either activates or inhibits adenylate cyclases in order to induce proper physiological responses. A key event in this signalling system is the direct and dynamic interaction of Gαi1 subunits with adenylate cyclases. We established a FRET-based assay between G-protein subunits and AC5 (type V adenylate cyclase) and monitored receptor-stimulated interactions between Gαi1 and AC5 in single intact cells with high temporal resolution. We observed that FRET between Gαi1 and AC5 developed at much lower concentration of agonist compared with the overall G-protein activity resulting in a left-shift of the concentration–response curve by approximately one order of magnitude. Furthermore, Gαi1-protein-mediated attenuation of AC5-dependent increases in cAMP occurred at comparable low concentrations of agonist. On analysing the dynamics we found the dissociation of the Gαi1 subunits and AC5 to occur significantly slower than the G-Protein deactivation and to be insensitive to RGS4 (regulator of G-protein signalling type 4) expression. This led us to the conclusion that AC5, by binding active Gαi1, interferes with G-protein deactivation and reassembly and thereby might sensitize its own regulation.

Key words: adenylate cyclase, FRET, GIRK (G-protein-activated inwardly rectifying K⁺) G-protein-coupled receptor, Gαi, RGS4 (regulator of G-protein signalling type 4).

INTRODUCTION

The second messenger cAMP regulates cellular functions in virtually all cells. Pharmacological interventions, aimed to treat diseases such as hypertension, asthma, diabetes, pain or Alzheimer’s disease [1], exert their effect via the alteration of cAMP. The amount of cAMP is controlled through its generation by ACs (adenylate cyclases) and the degradation by PDEs (phosphodiesterases) respectively. Several AC isoforms are under the dual control of stimulatory and inhibitory G-proteins [2,3]. Among the nine isoforms of membrane-spanning ACs, AC5 (type V AC) and AC6 (type VI AC) are most closely related [4]. AC5 is ubiquitously expressed at least on the mRNA level [5]. In the central nervous system AC5 is important for learning [6,7] and a major mediator of morphine action [9]. In the cardiovascular system, deletion of AC5 in mice leads to protection from cardiomyopathies [10,11]. Structures from protein crystals exist only for the catalytic domain C2 of AC5. AC5 is a large intracellular loop between the transmembrane helices 6 and 7, C2 is part of the intracellular C-terminus. These structures revealed information about binding sites for the nucleotide [12] and catalytically necessary cations [13], forskolin [14] and its analogues [15]. In addition they uncovered a mechanism for the regulation by Ca²⁺ [16] and the interaction with Gαi [12]. Further interaction sites for G-protein subunits have been determined by biochemical approaches. AC5 generation assays of cAMP suggest that catalytic domains in combination with chimaeric and mutated proteins revealed that Gαi1 binds to the C1 domain [17,18].

Pull-down experiments identified the catalytic domain C2 as the binding region for Gαi [19]. Finally, putative interaction sites for the Gβγ dimer and Gαi-GDP were mapped to the N-terminus of AC5 by pull-down and FRET studies [20]. However, the complete structure of the G-protein–AC complex remains elusive.

cAMP generation is a highly dynamic process and temporal patterns of cAMP signals are critically important for regulation of cell function (e.g. in pancreatic islets [21,22]). The development of FRET-based assays for detection of cAMP [23] provided a tool for kinetic imaging of cAMP levels in intact cells. Whereas the dynamics of G-protein-mediated regulation of ACs have so far been unknown, we and others resolved the kinetics of signalling events upstream of ACs, such as receptor and G-protein activation [24–27]. Ligand-induced receptor activation and interaction between receptor and G-protein occur rapidly (~40 ms). G-protein activation is slower, but still in the subsecond time scale [24,27], whereas receptor-mediated regulation of cAMP levels is much slower (t1/2 [time to half-maximal deactivation] ~30 s [27]). The present study aimed to delineate the dynamics of Gαi-mediated regulation of AC5. In particular, we wanted to investigate whether the reported high sensitivity of AC5 towards Gi-mediated inhibition [28] is reflected on the level of Gαi1–AC5 interaction and if so, how G-protein dynamics are affected. Therefore we established a FRET-based assay to monitor interactions between fluorescent Gαi subunits and AC5 in single intact cells. With this assay we aimed to address general questions regarding the impact of G-protein–effector interactions on the balance of the G-protein cycle and the ability of RGS (regulator of G-protein signalling) proteins to accelerate the dissociation of effector and

Abbreviations used: AC, adenylate cyclase, AC5, type V adenylate cyclase; α2A-AR, α2C-AR, α2-adrenocceptor; β1-AR, β2-adrenocceptor; CHO, Chinese-hamster ovary; CNG, cyclic nucleotide-gated; Epac, exchange protein directly activated by cAMP; GIRK, G-protein-activated inwardly rectifying K⁺; HEK, human embryonic kidney; HRP, horseradish peroxidase; Iso, isoprenaline; mYFP, membrane-bound YFP; NE, noradrenaline (norepinephrine); PDE, phosphodiesterase; RGS, regulator of G-protein signalling; t1/2, time to half-maximal deactivation; wt, wild-type; TBST, TBS with Tween 20.

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G-protein subunits. With the present study, we hoped to gain some mechanistic insight into general principles of how the same family of G-proteins regulates effectors with very different sensitivity even within the same cell [29].

**MATERIALS AND METHODS**

**Plasmids**

pcDNA3 YFP-hAC5 was kindly provided by Carmen W. Dessauer (Department of Integrative Biology and Pharmacology, University of Texas, Houston, TX, U.S.A.). Plasmids encoding \( \alpha_{2A} \)-AR (\( \alpha_{2A} \)-adrenoceptor), G\( \beta \)-\( \gamma \)-wt (wild-type), G\( \gamma \)_\( \alpha \)-CFP, G\( \gamma \)_\( \gamma \)-wt and G\( \alpha \)_\( \gamma \)-Cyt were used as described previously [24], as well as CD86–YFP [30], RGS4 [31], Epac1 (exchange protein directly activated by cAMP)-camps (cAMP sensor) [23] and membrane-associated YFP [26]. G\( \alpha \)_\( \gamma \)-CFP was cloned analogous to G\( \alpha \)_\( \gamma \)-YFP. The plasmid mGFP-10-sREACH N3, which contains a non-fluorescent YFP as described previously [32], was purchased from http://www.addgene.org/ (plasmid #21947).

**Cell culture and transfection**

HEK (human embryonic kidney)-293T cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were transfected using Qiagen Effectene Transfection Reagent according to the manufacturer’s instructions. Transfections were stopped with normal culture medium supplemented with 100 nM yohimbine to prevent serum activation of the \( \alpha_{2A} \)-AR. At 30 h after transfection the cells were split on coverslips coated with poly-l-lysine to obtain single cells for the measurement. For the G\( \alpha \)/G\( \beta \)/G\( \gamma \)-FRET assay, cells were transfected with 0.1 mg of \( \alpha_{2A} \)-AR, 0.4 mg of G\( \alpha \)_\( \gamma \)-YFP, 0.2 mg of G\( \beta \)_\( \gamma \)-wt, 0.1 mg of G\( \gamma \)_\( \gamma \)-CFP and 0.25 mg of RGS4 where indicated. For the G\( \alpha \)/AC5-FRET assay, cells were transfected with 0.1 mg of \( \alpha_{2A} \)-AR, 0.4 mg of G\( \alpha \)_\( \gamma \)-CFP, 0.2 mg of G\( \beta \)_\( \gamma \)-wt, 0.1 mg of G\( \gamma \)_\( \gamma \)-CFP, and 0.3 mg of YFP–AC5 and 0.25 mg of RGS4 where indicated. For the functional experiments 0.1 mg of \( \alpha_{2A} \)-AR, 0.25 mg of Epac1-camps and 0.3 mg of AC5-wt or sREACH–AC5 were used, as indicated in the Figures. To measure GIRK (G-protein-activated inwardly rectifying K\(^{+}\)) currents, cells were transfected with 1 mg of \( \alpha_{2A} \)-AR and 1 mg of GIRK1/4 (bicistronic vector [31]). All transfection mixes were adjusted with empty pcDNA3 to a total amount of 1.35 mg of DNA.

**Cloning of human YFP-AC5 and sREACH-AC5**

YFP-AC5 was cloned using the 3-fragment MultiSite Gateway® Pro system (Invitrogen). The 3-fragment system was chosen to allow for easy addition of fluorophores to either terminus of AC5. Primer design and reactions were carried out according to the manufacturer’s instructions. Four additional amino acids were added to the linker between fragment 1 and 2 (YFP/sREACH and AC5) to increase the linker’s flexibility. pcDNA3 YFP-AC5 was kindly provided by Carmen W. Dessauer and used as a template to amplify AC5 and thereby generate the entry clone for the second/middle fragment. From the plasmid mGFP-10-sREACH-N3 [32], sREACH was subcloned into pcDNA3 and amplified to generate the N-terminal fragment. The final linker sequence between YFP/sREACH and AC5 is AGAGHPTFLKYVAT. The C-terminal linker contains the stop codon and has the amino acid sequence TTLYNKVV. The entry clones for AC5 and YFP/sREACH were verified by sequencing. The final constructs were controlled by restriction digestion and partially sequenced.

**FRET measurements**

FRET measurements of transiently transfected HEK-293T cells were performed approximately 48–54 h after transfection at room temperature (20–24°C) using an inverted microscope (eclipse Ti, Nikon) equipped with a 100× oil immersion objective (Plan Apo VC 100×/1.40 oil ∞/0.17 Dic N2, Nikon). A fast-switching xenon arc-based illumination system (Lambda DG-4, Sutter Instrument) was used as the light source. The following filters (all from Chroma) were used: ET 430/24 (CFP excitation) or ET 500/20 (YFP excitation), T455LP (long-pass beam splitter to collect combined fluorescence of CFP and YFP) or CFP/YFP beam splitter plus CFP/YFP emission filter (catalogue numbers 59017bs and 59017mg, z488/800–1064rp (beam splitter to separate CFP and YFP emission), ET 480/40 (CFP emission) and HC 534/20 (YFP emission). The last three components were set in an Optosplit II (Cairn Research) to simultaneously record CFP and YFP fluorescence using a fast CCD (charge-coupled-device) camera (Evolve512, Roper Scientific). Microscope, camera and DG-4 were controlled using NIS-Elements AR (Laboratory Imaging). In order to synchronize the camera and lamp, an additional trigger-box was supplied by Nikon. Cells were continuously superfused with buffer (137 mM NaCl, 5.4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\) and 10 mM Hepes, pH 7.3) or buffer containing agonist in different concentrations using a fast-switching eight-channel valve-controlled pressurized perfusion system with solenoid valves (Ala-VC®-8SP, ALA Scientific Instruments). For FRET measurements CFP and YFP emission were recorded simultaneously while cells were excited with 430 nm light. Depending on the fluorescence intensity, the illumination time was set to 20–40 ms at an interval of 500 ms or 2 s in the Epac1-camps experiments. The lamp was set to lowest intensity to prevent bleaching. Cell fluorescence was recorded at 488 ± 20 nm (F\(_{\text{exc}}\) for CFP) and 534 ± 10 nm (F\(_{\text{534}}\) for YFP) and corrected for background fluorescence, resulting in F\(_{\text{CFP}}\) and F\(_{\text{YFP}}\). To determine F\(_{\text{RFP}}\), F\(_{\text{YFP}}\) was additionally corrected for bleed-through of CFP fluorescence into the F\(_{\text{534}}\) channel and direct excitation of YFP at 430 ± 12 nm excitation was subtracted. The resulting fluorescence was divided by F\(_{\text{CFP}}\) and F\(_{\text{YFP}}\), respectively, to refer to as the ‘FRET ratio’.

**Electrophysiology**

Patch pipettes (resistance 2–5 MΩ) were manufactured using borosilicate glass capillaries (GC150F-10, Harvard Apparatus) with a horizontal pipette puller (P87, Sutter Instruments). During experiments, cells were continuously superfused with extracellular buffer consisting of 20 mM KCl, 122 mM NaCl, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), and 10 mM Hepes (pH 7.4 with NaOH). The intracellular solution was composed of 100 mM K\(^{+}\)-aspartate, 40 mM KCl, 5 mM NaCl, 7 mM MgCl\(_2\), 2 mM EGTA, 0.25 mM GTP, 5 mM Na\(^{+}\)-ATP and 20 mM Hepes (pH 7.2 with NaOH). GIRK channels of HEK-293T cells expressing \( \alpha_{2A} \)-AR and GIRK1/4 subunits were activated by application of NE [noradrenaline (norepinephrine)]-containing solutions. Corresponding whole-cell GIRK currents were recorded in the inward direction (\( V_{\text{hold}} \): −90 mV, calculated EK: −48 mV) using an EPC 7 Amplifier with an ITC-16 interface and Patchmaster software version 2.52 (all from HEKA). To identify...
GIRK currents, background-subtracted $I/V$ relationships were obtained by applying fast voltage ramps ($-120$ mV to $+60$ mV within 500 ms) in the absence and presence of NE.

**Donor recovery after acceptor photobleaching**

Cells were kept in buffer without agonist during the bleaching process. Making use of the CFP/YFP filters described above we collected CFP fluorescence during a 6-min bleaching process for YFP. Fluorescence was recorded every 5 s. Between recordings the lamp was set to permanent YFP excitation ($500 \pm 10$ nm) at the highest possible intensity to bleach the FRET acceptor. The relative $F_{\text{CFP}}$ change before $(F_{\text{CFP,0}})$ and after bleaching $(F_{\text{CFP}})$ was evaluated as $\frac{F_{\text{CFP,0}} - F_{\text{CFP}}}{F_{\text{CFP,0}}}$. 

**Quantification of relative expression levels by means of fluorescence**

We used the construct YFP–$\beta_2$–AR–CFP as published previously [30] for calibration of the stoichiometry of relative expression levels of CFP and YFP. This construct bears the same fluorophores used in YFP–AC5 and Gαi1–CFP, which allows for fluorescence comparison. The fluorescence intensities for both fluorophores, individually excited, were recorded and corrected for background fluorescence. $F_{\text{CFP}}$ was divided by $F_{\text{YFP}}$ to calculate the calibration factor. To calculate the individual expression ratio, the $F_{\text{CFP}}/F_{\text{YFP}}$ ratio of the Gαi1/AC5-FRET cells was measured similarly and was divided by the calibration factor to determine the amount of Gαi1–CFP overexpression over YFP–AC5.

**Data evaluation**

All FRET recordings were corrected for photobleaching by subtracting a monoexponential baseline using OriginPro (OriginLab), unless stated otherwise. Signal amplitudes were calculated as agonist-induced alteration of the FRET signal $\Delta(F_{\text{YFP}}/F_{\text{CFP}})$. As the Gαi1/AC5-FRET recordings could not be fitted properly to simple exponential equations, we determined $t_{0.5}$ values directly from the traces. In all cases $t_{0.5}$ was determined as the time to reach half of the maximally evoked FRET amplitude after agonist exposure or withdrawal. Application of saturating concentrations of NE resulted in an additional increase in the FRET ratio of the Gαi1/AC5-FRET after agonist withdrawal before recovering to baseline as shown in Figures 2, 5 and 6. Interestingly, this transient increase resulted in an amplitude comparable with the FRET change of the highest concentration that did not show this effect (usually a concentration below 10 nM NE). Increasing concentrations of NE reduced the actual amplitude of the agonist-induced FRET increase, but did not affect the total amplitude of the transient. We therefore hypothesized an inhibitory effect of the receptor as reported previously [33]. In the concentration–response experiments we measured the peak value of this transient as the total amplitude of the agonist-induced FRET change at the given concentration. Most likely owing to desensitization effects we sometimes observed an additional reduction in the total amplitude for 1 μM NE in the Gαi1/AC5-FRET, resulting in bell-shaped concentration–response curves (see Figure 3). In those experiments we normalized to the next lower concentration and omitted the 1 μM NE value during sigmoidal fitting.

**Western blotting of HA-tagged $\alpha_{2\mathrm{A}}$–AR**

Transiently transfected HEK-293T cells were transferred from 6-cm-diameter dishes to 10-cm-diameter dishes 24 h after transfection and incubated in culturing medium with 100 nM yohimbine for another 24 h. The medium was removed and the cells immediately frozen at $-80^\circ$C. To lyse the cells, the dishes were thawed on ice, 1 ml of lysis buffer [20 mM Tris, 2 mM EDTA and protease inhibitor mix (Complete ULTRA Tablets Mini EDTA-free, EASYpack; Roche)] was added, cells were scraped and then resuspended in buffer. The suspensions were homogenized for 30 s with an IKA T10 basic (Ultra-Turrax). The protein amount was determined using Bradford’s reagent (Applichem). The lysates were adjusted with lysis buffer to equal concentrations of protein. To denature the samples five loads of sample buffer [50% (w/v) glycerine, 312.5 mM Tris/HCl (pH 6.8), 10% (w/v) SDS, 25% (w/v) 2-mercaptoethanol and 0.1% Bromophenol Blue, in water] was added and samples were heated to 95°C for 15 min. The lysates were separated with SDS/PAGE (10% gel). Proteins were transferred on to a PVDF membrane (Roche) with wet blotting. The membrane was blocked with 5% (w/v) non-fat dried skimmed milk powder [in TBST (TBS with Tween 20)] for 1 h at room temperature and incubated with the primary antibody [purified HA.11 clone 16B12 monoclonal antibody (Covance); 1:500 dilution] overnight at 4°C. A HRP (horseradish peroxidase)-linked horse anti-mouse antibody (Vector Laboratories; 1:4000 dilution) was used as secondary antibody. The membrane was incubated for 90 s with HRP-Juice PLUS (PJ) and bioluminescence was detected and recorded using a Chemidoc system (Bio-Rad Laboratories). To check for equal loading the membrane was stripped with stripping buffer [1.5% (w/v) glycine, 0.1% SDS and 1% (v/v) Tween 20 (pH 2.2)] for 15 min, washed with PBS and TBST (5 min each) and blocked again with non-fat dried skimmed milk powder for 1 h. The primary antibody against actin [actin clone C4 (MB Biomedicals); 1:100000 dilution] was incubated overnight and detected with the same secondary antibody as described above.

**Western blot analysis**

Blots acquired with ChemiDoc™ were analysed using ImageJ (1.46r; http://image.nih.gov/ij/). A rectangular-shaped region of interest was placed on each band and raw intensities were measured. An additional region of interest of the same size was placed over an empty part of each band to allow for background correction. The obtained intensity values were analysed with Excel 2010 (Microsoft).

**Bioluminescent cAMP assay**

The cAMP assay has been described previously [34]. In general, this assay uses aequorin with its cofactors coelenterazine and Ca2+ to generate bioluminescence. The activation of ACs results in an increase in cAMP, which in turn opens CNG (cyclic nucleotide-gated) channels. Subsequently Ca2+ can enter the cells, which are kept for a brief period in Ca2+-free tyrode. The CNG channel is opened according to the amount of generated cAMP, hence increasing amounts of cAMP will result in a higher portion of open channels, which in turn increases the Ca2+ influx and yields higher bioluminescence.

**Software and statistics**

To evaluate fluorescence recordings, we used Excel 2010 (Microsoft), OriginPro 8.6 (OriginLabs) and GraphPad Prism 5. Statistics were obtained by Student’s $t$ test, Dunnet’s multiple comparison test or ANOVA with post-hoc tests as indicated in the individual Figure legends. ImageJ (1.46r) and Corel Photo Paint X4 (Corel) were used to process the confocal images in Figure 1(A).
RESULTS AND DISCUSSION

In order to analyse the interaction between G-protein subunits and AC5 in living cells, we cloned a variant of eYFP [enhanced YFP (F46L/L68V)] to the N-terminus of AC5 similar to a construct reported previously [20]. YFP–AC5 localized to the membrane when transiently expressed in HEK-293T cells as depicted in Figure 1(A). Functionality of YFP–AC5 with respect to G-protein-mediated stimulation was proven by a bioluminescence-based cAMP generation assay which relies on CNG channel Ca2+ influx (see [34] for a detailed description of the assay). Both expression of AC5-wt and YFP–AC5 resulted in a robust increase in cAMP on Iso (isoprenaline)-induced stimulation of β2-ARs (β2-adrenoceptors) in comparison with control cells transfected with mYFP (membrane-bound YFP) (Figure 1B). The Iso-dependent increase in cAMP in the control cells can be attributed to endogenous ACs in CHO (Chinese-hamster ovary) cells. From the results of the present study, we concluded YFP–AC5 to be fully competent for signalling with regard to Gβγ-mediated stimulation. Further evidence for functional regulation through Gαi- and Gβγ-proteins was drawn from cAMP experiments as shown in Figure 6(C) and Supplementary Figure S1 (at http://www.biochemj.org/bj/454/bj4540515add.htm). Not only was the stimulation via Gαi-proteins remarkably similar for tagged and untagged AC5, but also the inhibition via endogenous Gαi-proteins. Owing to the lack of suitable antibodies against AC5 we could neither determine expression levels nor subcellular distribution of endogenous or transfected AC5. This problem has been reported previously [35].

Dynamic interaction between YFP–AC5 and Gβγ–CFP

In order to measure the kinetics of interactions between G-proteins and ACs in an agonist-dependent manner we established a FRET-based assay in single HEK-293T cells. These were co-transfected with YFP–AC5 and the previously described Go13–CFP [26] together with the Gαβγ subunits (hereafter referred to as Gα/AC5–FRET). Single living cells were superfused with either buffer or NE-containing buffer to activate the Gαβγ-AR and the Gi signalling pathway in this and all of the following experiments. Application of NE resulted in an agonist-dependent reversible increase in FRET ratio between Go13–CFP and YFP–AC5 (Figure 2A), that reached a plateau after approximately 25 s at ∆(F_{cFP}/F_{oFP}) = 0.018 ± 0.003 (mean ± S.E.M.) (Figures 2B and 5B). A limited amplification for the relatively small amplitude of the agonist-evoked FRET signal might be a distance between the fluorophores larger than the Förster radius. To verify the specificity of the agonist-induced FRET change, we checked for unspecific interaction between the G-protein and other membrane proteins. The CD86 T-cell receptor supposedly does not interact with the agonist-evoked FRET increase, the control condition is hardly affected at all. (C) Changes in donor fluorescence after acceptor photobleaching. HEK-293T cells were transfected with labelled proteins as indicated together with receptor and G-protein subunits. The acceptor (YFP) was bleached for 6 min and the relative change of CFP fluorescence is depicted as mean ± S.E.M. for at least nine cells out of three to four transfections. Statistics in (B) and (C) were obtained using Student’s t test with Welch correction or ANOVA and Dunnett’s multiple comparison test against Go13–CFP/CD86–YFP control respectively (***)P < 0.001).

Figure 1 Functional characterization of YFP–AC5

(A) Subcellular distribution of YFP–AC5 and Go13–CFP in transiently transfected HEK-293T cells together with unlabelled α2A-AR and Gβγ2. Both fluorophores were excited separately. (B) Average concentration–response curves to show Gi signalling competence of wild-type and YFP–AC5–Gβγ2 subunits. G-proteins were stimulated via activation of α2A-AR with 10 nM NE, resulting in an increase in the FRET ratio (upper panel, black trace), which is confirmed by the opposing movement of the individual fluorescence traces. This trace has not been corrected for photobleaching. (B) The agonist-induced change in the FRET ratio of at least eight cells for each condition is depicted as mean ± S.E.M. Although the Go13/AC5-FRET pair shows a robust agonist-dependent FRET increase, the control condition is hardly affected at all. (C) Changes in donor fluorescence after acceptor photobleaching. HEK-293T cells were transfected with labelled proteins as indicated together with receptor and G-protein subunits. The acceptor (YFP) was bleached for 6 min and the relative change of CFP fluorescence is depicted as mean ± S.E.M. for at least nine cells out of three to four transfections. Statistics in (B) and (C) were obtained using Student’s t test with Welch correction or ANOVA and Dunnett’s multiple comparison test against Go13–CFP/CD86–YFP control respectively (***)P < 0.001).

Figure 2 Interaction between Go13–CFP and YFP–AC5

(A) Representative recording of CFP and YFP emission (lower panel, dark and light grey trace respectively) upon CFP excitation derived from a single HEK-293T cell transfected with Go13–CFP, YFP–AC5, Go13–CFP and Go13–AR and Gβγ2 subunits. G-proteins were stimulated via activation of α2A-AR with 10 nM NE, resulting in an increase in the FRET ratio (upper panel, black trace), which is confirmed by the opposing movement of the individual fluorescence traces. This trace has not been corrected for photobleaching. (B) The agonist-induced change in the FRET ratio of at least eight cells for each condition is depicted as mean ± S.E.M. Although the Go13/AC5-FRET pair shows a robust agonist-dependent FRET increase, the control condition is hardly affected at all. (C) Changes in donor fluorescence after acceptor photobleaching. HEK-293T cells were transfected with labelled proteins as indicated together with receptor and G-protein subunits. The acceptor (YFP) was bleached for 6 min and the relative change of CFP fluorescence is depicted as mean ± S.E.M. for at least nine cells out of three to four transfections. Statistics in (B) and (C) were obtained using Student’s t test with Welch correction or ANOVA and Dunnett’s multiple comparison test against Go13–CFP/CD86–YFP control respectively (***)P < 0.001).
resulted in an increase in Gγ–CFP fluorescence of approximately 5%. Acceptor photobleaching of the Goαi1–CFP/YFP–AC5-FRET pair resulted in a decrease in donor fluorescence of approximately 2%. A similar decrease in CFP fluorescence was observed in cells transfected with Goαi1–CFP and AC5-wt, α2A-AR and Gβγ. This condition was used to control for photobleaching of the donor during the experiment. Furthermore, comparable results were obtained in cells expressing Goαi1–CFP and CD86–YFP as negative control. Taken together, these results suggest no basal, but agonist-dependent, interaction between Goαi1–CFP and YFP–AC5. We consider the observed agonist-evoked FRET signals to be specific, on the basis of their kinetics and reversibility, as well as their absence following replacement of YFP-AC5 with the membrane targeted CD86–YFP receptor. The results from the acceptor bleaching experiments do not support the concept of preformed complexes that have been reported previously by others [36]. However, basal interaction between AC5 and the Gi-protein cannot be ruled out either, since the absence of a specific FRET signal could also be attributed to an unfavourable orientation or distance of the fluorophores.

Interaction between Goαi1–YFP and YFP–AC5 shows very high sensitivity

In order to test whether the interactions between AC5 and Goαi1 themselves affect dynamics and the equilibrium of G-protein activation we compared agonist-evoked FRET signals between Goαi1–CFP and YFP–AC5 with those between Goαi1–YFP and Gβ1 and CFP–γ2. The latter resolves G-protein subunit rearrangements upon receptor-mediated stimulation, reflecting G-protein activation as reported previously (Figure 3A) (see also [24,37]). Both fluorescent G-protein subunits were fully functional with respect to receptor and effector coupling [24,37]. In the following, the Goαi1–YFP/Gγ–CFP assay will be referred to as Goαi1–Gγ–FRET. Activation of Gγ-coupled receptors leads to an increase in the FRET ratio between Goαi1–YFP and Gγ–CFP within seconds [24] (Figures 3A and 4A, upper trace), indicating G-protein activation. As discussed previously, this increase in FRET argues against the hypothesis of complete subunit dissociation upon activation of the Gγ-protein, but rather argues for rearrangement of the subunits relative to each other [24,37]. To keep the experimental conditions as similar as possible we used equal amounts of plasmids for the Goαi1–AC5- and Goαi1–Gγ–FRET assay. Comparing concentration–response curves for both FRET assays, we were surprised to detect a significantly higher sensitivity of the agonist-evoked Goαi1–AC5 interaction than for the Goαi1–Gγ–FRET interaction. The respective EC50 values were 0.3 nM NE and 3.2 nM NE. This 10-fold shift was highly significant (Figure 3B). Since previous studies had shown a clear correlation between α2A-AR expression level and signalling potency [38], we tested whether different expression levels of the α2A-AR could account for the shift in the concentration–response. Therefore we performed Western

Figure 3 Sensitized interaction between Goαi1 and AC5 in contrast with G-protein activation

(A) Representative ratiometric FRET imaging of a single HEK-293T cell transfected with Goαi1–YFP, Gβ1, CFP–γ2 and α2A-AR. This trace has not been corrected for photobleaching. The lower panel shows the individual fluorescence traces, with FCFP being presented in darker grey than FYFP. Activation of the G-protein via the α2A-AR by 1 μM NE results in an increase in the FRET ratio (upper panel) as reported previously [24]. (B) Representative concentration–response curves (upper panel) derived from single cells for NE-evoked Goαi1/Gβγ– or Goαi1/AC5-FRET with or without co-expression of RGS4 as indicated. The x-axis of the lower panel also applies to these graphs. Details of data analysis and curve fitting are described in the Materials and methods section. The lower panel shows the mean EC50 values derived from fittings of individual curves for the indicated conditions (mean ± S.E.M.; n>11) (statistics were obtained by ANOVA and Bonferroni post-hoc test; ***P < 0.001, n.s., not significant). (C) Western blot to analyse the expression of the α2A-AR in HEK-293T cells transfected with plasmids for Goαi1/Gγ/FRET (Goαi1/AC), Goαi1/Gβγ/FRET (Goαi1/Gβγ–FRET) or the assay for Goαi1/AC5-FRET (Goαi1/AC). Transfections were performed independently on three different days as indicated by the brackets. (D) The fluorescence intensity of either Goαi1–CFP or YFP–AC5 was measured individually, corrected as detailed in the Materials and methods and the Results and discussion section and is depicted as the ratio of CFP/YFP (mean ± S.E.M.; n = 15). This represents the expression of Goαi1 in relation to AC5. On average, both constructs are expressed equally, with a tendency of YFP–AC5 being expressed stronger than Goαi1–CFP.
bliot analysis against the HA-tag on the receptor, but did not detect a higher expression of the α2A-AR in the presence of AC5 (Figure 3C). As strong overexpression of Gαi1-CFP over YFP-AC5 could lead to a saturation-based apparent higher sensitivity of the interaction, we quantified the membrane expression levels of both proteins by means of fluorescence intensities as detailed in the methods section. In our experiments, Gαi1-CFP and YFP-AC5 were nearly equally expressed. The ratio between Gαi1 and AC5 was 0.92 ± 0.13 (mean ± S.E.M.; n = 15) ranging from 0.3 to 2 (3-fold overexpression of AC5 over Gαi1 or 2-fold excess of Gαi1 over AC5 respectively) (Figure 3D). Therefore we can exclude that the majority of the observed increase in sensitivity of the agonist-evoked Gαi1-AC5 interaction in comparison with the Gαi1-Gβγ interaction was due to a strong overexpression of G-proteins relative to AC5. We hypothesized that this remarkable discrepancy in agonist sensitivity was caused by the direct interaction between the signalling partners. This led us to compare AC5 with other G1-protein effectors and analyse whether cAMP signals generated via AC5 could be functionally inhibited via Gi1 at very low agonist concentrations.

**AC5 is much more sensitive to receptor-mediated G1-activation than G1R channels**

In order to verify that the observed sensitization towards receptor-mediated regulation is a feature of the interaction between Gαi1 and AC5, we used the G-protein-gated GIRK channel as a non-AC-related effector under similar experimental conditions. This Gβγ effector has been demonstrated previously to exhibit a similar sensitivity towards α2A-AR-mediated stimulation as the Gαi1/Gβγ-FRET assay [24]. NE (0.3 nM) only activated GIRK currents at a fraction of 0.11 ± 0.03 (mean ± S.E.M.; n = 6) of the maximum (Figures 4A and 4B). This closely resembles the value of the Gαi1/Gβγ-FRET assay (compare Figure 3A with Figures 4A, 4B and 4D). In general, the concentration–response of the GIRK channel resembled that of the Gi1-protein itself, the EC50 being 19 nM NE (Hill coefficient of 0.9) (Figure 4D).

To directly measure cAMP, we used the FRET-based cAMP sensor Epac1-camps [23] which allows time-resolved detection of cellular cAMP levels. Considering the dynamic range of this sensor we worked out a protocol suitable to study Gαi-mediated inhibition of cAMP signals generated via AC5 as described below. HEK-293T cells were transfected with Epac1-camps, AC5-wt and the α2A-AR (or empty vector for control purposes). Making use of the endogenously expressed β2-AR we stimulated the generation of cAMP by continuously superfusing the cells with 3 nM Iso which led to a decline in the Epac1-camps FRET signal, reflecting a rise in intracellular cAMP. This rise was much more robust in the case of AC5-expressing cells (Figure 4C). During this Iso application we added NE at different concentrations. Only cells transfected with the α2A-AR showed a strong inhibition of the Iso-evoked cAMP generation as indicated by the rise in Epac1-camps FRET ratio shown in Figure 4C. In the case of AC5-expressing cells, stimulation of α2A-ARs with 0.3 nM NE completely reversed the β2-AR-evoked rise in cAMP and even reduced the basal (non-agonist-dependent) levels of cAMP, indicated by a rise in the FRET ratio to levels above baseline values before stimulation with Iso. Comparison with cells transfected with empty vector instead of AC5, revealed the AC5 dependency for both the β2-AR-mediated rise in cAMP and its α2A-AR-mediated attenuation (Figure 4D). To measure concentration–response curves we applied increasing concentrations of NE while continuously stimulating with 3 nM Iso. The difference in FRET between the plateau of the FRET increase mediated by 3 nM NE and the lowest plateau caused by 3 nM Iso was used to normalize the amplitude of the other concentrations. The resulting concentration–response curve has an EC50 of 0.08 nM NE (Hill coefficient of 5.9) (Figure 4D). This value is even further left-shifted compared with the interaction of Gαi1 and AC5 (Figure 3B) and the activity of G1-protein and GIRK channel (Figures 3B and 4D). In addition the curve is rather steep, which most likely can be attributed to the amplification mechanisms, limited detection range of the Epac1-based sensor and PDE activity.

A similar difference in agonist sensitivity between cAMP-dependent and G1-protein activity-mediated signalling outcomes was reported by Li et al. [29], who compared the regulation of L-type Ca2+ currents and GIRK currents following activation of M2-ACh (muscarinic subtype 2 acetylcholine receptor) in adult cardiac myocytes. At lower concentrations of acetylcholine they found a Gαi1-dependent inhibition of L-type Ca2+ currents (via inhibition of AC and a reduction in cAMP levels), whereas higher concentrations also resulted in a G1-mediated activation of GIRK channels.

![Figure 4 Comparison of the sensitivity of G1-dependent regulation of GIRK channel and AC5](image-url)

(A) Representative recording of agonist-induced GIRK currents. The cell was stimulated with the indicated concentrations of NE. Between the stimulations, the cell was superfused with agonist-free buffer to wash out the agonist and allow the currents to recover to baseline (not shown). The spikes in the trace are voltage ramps to generate the voltage–current plot for the channel. For a detailed description of the experiment see [41]. (B) Voltage–current relations of the NE-evoked currents generated from the recordings in (A). (C) HEK-293T cells were transfected with Epac1-camps, AC5-wt and α2A-AR where indicated in order to detect time-resolved cAMP signals in intact cells. Single-cell recordings were performed and the cells were superfused with Iso to stimulate cAMP generation, which results in a loss of FRET. Only in α2A-AR transfected cells the cAMP generation stimulated by 3 nM Iso was attenuated upon application of 0.3 nM NE. Cells that were not transfected with AC5-wt generally showed a smaller response to both Iso and NE. The recordings of eight to ten cells from three independent transfections were normalized to the baseline before Iso stimulation (mean ± S.E.M.; n = 8). (D) Concentration–response curves for GIRK channel activity (squares) and the NE-mediated inhibition of Iso-induced cAMP elevation (triangles). The GIRK channel activity at a given concentration was normalized to the maximum activity stimulated by 500 nM NE (mean ± S.E.M.; n = 5). The GIRK channel curve closely resembles that of the G1-protein activity depicted in Figure 3B. The cAMP experiments were performed similar to those depicted in (C), but with three increasing concentrations of NE, always including 3 nM NE. The respective FRET changes were normalized to the maximum FRET change evoked by 3 nM NE in comparison with the final FRET plateau of 3 nM Iso after NE withdrawal (mean ± S.E.M.; n = 7).
Our hypothesis that the interaction of AC5 with Ga_{i1} itself causes the sensitization of AC5 towards Gi-mediated inhibition was derived from observations made with YFP–AC5. Therefore we wanted to compare the fluorescently labelled AC5 with AC5 wt. To avoid interference of the YFP fluorescence of YFP–AC5 with Epac1-camps FRET signals, we mutated the chromophore of wt. To avoid interference of the YFP fluorescence of YFP–AC5 with Epac1-camps FRET, we wanted to compare the fluorescently labelled AC5 with AC5-wt and sREACh–AC5. Compared with AC5-wt, sREACh–AC5 show equal kinetics and amplitudes of cAMP generation as monitored by Epac1-camps (Supplementary Figure S1) of the NE-mediated inhibition of the Iso-induced loss of Epac1-camps-FRET. Together with evidence presented in Figure 1, we conclude that YFP–AC5 is comparable with AC5-wt and sREACh–AC5. Therefore we conclude that at least the enzymatic activity is not influenced by the label on AC5.

Figures 3(C) and 3(D) show that the sensitivity is not influenced by either receptor expression levels or overexpression of the FRET donor. The simplest plausible explanation for the increased sensitivity of the Ga_{i1}/AC5 interaction would therefore be that the off-rate of the AC5–G-protein interaction is slowed relative to the Ga_{i1}/AC5–G-protein interaction with AC5-wt and sREACh–AC5. Compared with AC5-wt, sREACh–AC5 reached 95.6 ± 6.1 % (mean ± S.E.M.; n = 10) (Supplementary Figure S1) of the NE-mediated inhibition of the Iso-induced loss of Epac1-camps-FRET. Together with evidence presented in Figure 1, we conclude that YFP–AC5 is comparable with AC5-wt and sREACh–AC5 show equal kinetics and amplitudes of cAMP generation as monitored by Epac1-camps (Supplementary Figure S1). We therefore conclude that at least the enzymatic activity is not influenced by the label on AC5.

Interaction between Ga_{i1}–YFP and YFP–AC5 is prolonged and not affected by RGS4

We noticed that FRET between Ga_{i1}–CFP and YFP–AC5 (Figure 2A) recovered more slowly than we would have expected from G-protein activation/deactivation assays published previously [24,37]. The onset kinetics of Ga_{i1}–protein activation, as resolved by the Ga_{i1}/Gβγ–FRET assay, were similar to those observed in the Ga_{i1}/AC5–FRET assay (Figure 5A, lower trace, and Figure 5B). This was particularly obvious when the averaged FRET traces for both conditions were scaled to their respective maximal amplitude (Figure 5B, black squares compared with grey triangles). In contrast, upon agonist withdrawal, the FRET signal detecting interactions between Ga_{i1} and AC5 declined more slowly than that reflecting G-protein deactivation (Figures 5A and 5C). At saturating agonist concentrations we consistently observed a small and transient increase in the FRET ratio of the Ga_{i1}/AC5–FRET following agonist withdrawal (see the Materials and methods section for a further description).

On the basis of these results we hypothesized that AC5 interacts with Ga_{i1}–GTP which would result in prolongation of the active state of the Ga_{i1}–protein as reflected by its prolonged interaction with AC5. To investigate this further we tried to accelerate G-protein deactivation by stimulating GTP hydrolysis. RGS4 has been demonstrated to be very efficient in enhancing the endogenous GTPase activity of the Ga_{i1}–subunits, thereby accelerating G-protein deactivation in intact cells [40]. When co-expressed together with the Ga_{i1}/Gβγ–FRET assay, RGS4 markedly accelerated the Ga_{i1}–protein deactivation (Figure 6). In total, RGS4 accelerated the deactivation of the Ga_{i1}–protein approximately 2-fold and reduced the t_{1/2} from 29.3 ± 3.7 s to 15.8 ± 1.7 s (mean ± S.E.M.) (Figures 6B and 6C). In contrast, the t_{1/2} for the recovery of the Ga_{i1}/AC5–FRET back to baseline was not significantly affected by the co-transfection of RGS4 (t_{1/2} = 42.9 ± 2.7 s and 37.1 ± 3.6 s respectively; Figures 6B and 6C). These results indicate that the lifetime of the complex...
of Gαi and AC5 is not sensitive to RGS-dependent G-protein deactivation. Currently, we cannot determine whether the prolonged interaction between Gαi and AC5 is due to an AC5-dependent inhibition of the GTPase activity of the Gαi subunit or to a prolonged interaction with Gαi-GDP after GTP hydrolysis. Important biochemical studies that monitored Gαi-regulated cAMP generation of purified enzymes revealed that AC5 is regulated to a similar extent by both Gαi-GTP and Gαi-GDP [17]. Therefore on a functional level, the open question of whether the prolonged interaction between AC5 and Gαi occurs in the GTP- or GDP-loaded state of the G-protein might not be very important. RGS4 expression did not affect the kinetics of the Gαi/AC5-FRET which could either be due to an inability of RGS4 to increase the GTPase activity of the active AC5-bound Gαi subunit or a surprisingly slow dissociation of Gαi-GDP from AC5. In any way, the prolonged interaction of the two proteins delays the reassembly of Gαi-GDP and Gβγ to the inactive G-protein and thereby slows the G-protein cycle. This effect would explain the observed left-shift of concentration–response curves, because the equilibrium in the G-protein cycle is shifted towards a larger amount of AC5-bound G-proteins.

Acceleration of G-protein deactivation by RGS4 could be expected to lead to a right-shift of the concentration–response curves for receptor-mediated G-protein activation. However, we did not observe a significant reduction in the sensitivity of receptor-induced Gαi-protein activity, even though RGS4 tended to right-shift the EC50 values (Figure 3A, 0.8 nM NE and 6 nM NE respectively). This is in line with previous reports [40] and might be due to the ability of RGS proteins to accelerate not only offset, but also onset-kinetics, a phenomenon for which a mechanistic explanation is still under debate.

Apart from the interaction kinetics being a major contributor to the increased sensitivity of the Gαi/AC5-FRET in comparison with Gαi-protein activity, a second alternative mechanism could contribute to it. The observed transient increase in the FRET signal of the Gαi/AC5 assay after withdrawal of agonist (Figure 5B), specifically saturating concentrations of it, led to bell-shaped concentration–response curves. This agonist-induced inhibition at higher concentrations is possibly related to the previously reported Gαi/AR-mediated inhibition of G-protein activity [33]. It is important to exclude the possibility that the fluorescent tags alter the function of the proteins under investigation in a way that affects interpretation of the obtained results. In our experiments, AC5-wt and tagged AC5 altered cAMP levels on receptor activation in intact cells with indistinguishable kinetics. Furthermore, the high sensitivity of the interaction between fluorescent Gαi and AC5 was fully reflected in functional regulation of untagged AC5 via endogenous G-proteins. On the basis of previous studies, which rigorously tested functionality of tagged G-protein subunits with respect to both receptor and effector coupling [24] and the observed similarity of the concentration–response of the Gαi/Gβγ-assay and the GIRK current measurements (compare Figure 3B with 4D), we consider a reduced agonist sensitivity of the fluorescently labelled G-protein subunits to be highly unlikely.

As mentioned above, Li et al. [29] reported a higher sensitivity of agonist-induced Ca2+ current inhibition than GIRK channel activation. Furthermore, specific regulation of AC5 by dopamine D3 receptors was observed [28] and suggested a very sensitive inhibition of AC5 by Gαi-proteins. Both studies mainly used cAMP production or further downstream events as readouts. Therefore it remained unclear whether downstream regulatory events or even the stoichiometry of Gαi/AC5 expression influenced these measurements and the underlying mechanism of this increased sensitivity remained elusive. We demonstrate in the present study that the direct interaction between the two partners causes the sensitivity shift observed herein and previously. To our knowledge the present study provides the first direct evidence that the interaction of a G-protein effector with its G-protein can actually lead to a prolongation of the G-protein cycle of the effector-associated pool of G-proteins, thereby sensitizing its own receptor-induced regulation. It remains an open question whether AC5 would or could alter overall G-protein kinetics as assessed by the Gαi/Gβγ-FRET. Unfortunately, it is currently impossible to find appropriate experimental conditions under which AC5 is expressed in excess of fluorescent G-proteins, as overexpressed AC5 tends to accumulate in intracellular membranes and often is not trafficked properly to the cell membrane (this has also been reported previously [20]). Therefore it is currently not possible to address this open question.

Taken together, the present study shows that the previously observed higher sensitivity of cAMP-dependent over G-protein activity-dependent pathways [29] already occurs at the level of molecular interaction between AC5 and Gαi. The differences in interaction kinetics are a putative mechanism for the dramatically increased sensitivity of the Gαi-protein effector AC5 towards Gαi-dependent regulation. In contrast with the non-AC5-bound G-proteins, kinetics of Gαi/AC5 dissociation were not sensitive to RGS4. On the basis of these results we propose that the dynamics of G-protein/effector interactions might provide another fine-tuning mechanism for GPCR (G-protein-coupled receptor)-mediated signal transduction.

Figure 6  Influence of RGS4 on the kinetics of Gαi-protein deactivation and G-protein/AC5-dissociation

(A) Sample traces depicting the agonist-dependent change in the FRET ratio between Gαi and Gβγ. The FRET change was stimulated by activation of the co-transfected α2A-AR with NE. In the presence of RGS4 subunit, rearrangement of the G-protein is faster than without RGS4. The traces were normalized to the total amplitude of the agonist-evoked FRET change. (B) The agonist washout phase after 10 nM NE of at least 12 cells out of four to six transfections for each condition was normalized and averaged (mean ± S.E.M.). For better comparison, the dataset from Figure 5(C) was reproduced here. RGS4 accelerates the deactivation and rearrangement of the Gαi-protein in comparison with the RGS4-free condition. In contrast, RGS4 does not significantly affect Gαi/AC5 dissociation kinetics. The dissociation of Gαi and AC5 is generally slower than the rearrangement (reflecting deactivation) of the Gαi-protein subunits. (C) Presented are the t1/2 of the conditions shown in (B) (mean ± S.E.M.). The t1/2 was measured in the individual experiments and averaged. The Gαi-protein rearrangement takes approximately half as long as the Gαi/AC5 dissociation. RGS4 accelerates the Gαi-protein deactivation even more. Statistics were obtained using ANOVA with Bonferroni post-hoc test (∗P < 0.05, ⋆⋆P < 0.001, ns, not significant).
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AUTHOR CONTRIBUTION
Markus Milde cloned the plasmids, designed and performed the experiments and analysed the results. Andreas Rinne designed and performed electrophysiological experiments and contributed to the writing of the paper. Frank Wunder designed and performed the bioluminescent cAMP measurements. Stefan Engelhardt provided starting plasmids for the cloning strategy and contributed with important advice to the paper. Moritz Bünemann directed the project, designed the experiments and analysed the results. Markus Milde and Moritz Bünemann wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Dynamics of $G_{\alpha_{i1}}$ interaction with type 5 adenylate cyclase reveal the molecular basis for high sensitivity of $G_{i}$-mediated inhibition of cAMP production

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Figure S1 Comparison of wild-type and labelled AC5

HEK-293T cells were transfected with Epac1-camps, $\alpha_{2A}$-AR and either AC5-wt, sREACh–AC5 or pcDNA3 as indicated in order to detect time-resolved cAMP signals in intact cells. Single-cell recordings were performed and the cells were superfused with Iso to stimulate cAMP generation, which results in a loss of FRET. Only in $\alpha_{2A}$-AR transfected cells the cAMP generation stimulated by 3 nM Iso was attenuated upon application of 0.3 nM NE. Cells that were not transfected with AC5 generally showed a smaller response to both Iso and NE. The recordings of 10–11 cells from four to five independent transfections were normalized to the baseline before Iso stimulation (mean ± S.E.M.). Note that the curves for AC5-wt and sREACh–AC5 are superimposable, indicating similar function of the wild-type and tagged protein.

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