Use of a cAMP BRET Sensor to Characterize a Novel Regulation of cAMP by the Sphingosine 1-Phosphate/G_{13} Pathway*\textsuperscript{5}

Received for publication, October 16, 2006, and in revised form, January 29, 2007. Published, JBC Papers in Press, February 5, 2007. DOI 10.1074/jbc.M609695200

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Regulation of intracellular cyclic adenosine 3',5'-monophosphate (cAMP) is integral in mediating cell growth, cell differentiation, and immune responses in hematopoietic cells. To facilitate studies of cAMP regulation we developed a BRET (bioluminescence resonance energy transfer) sensor for cAMP, CAMYEL (cAMP sensor using YFP-Epac-RLuc), which can quantitatively and rapidly monitor intracellular concentrations of cAMP in vivo. This sensor was used to characterize three distinct pathways for modulation of cAMP synthesis stimulated by presumed G\textsubscript{i}-dependent receptors for isoproterenol and prostaglandin E\textsubscript{2}. Whereas two ligands, uridine 5'-diphosphate and complement C5a, appear to use known mechanisms for augmentation of cAMP via G\textsubscript{i}/calcium and G\textsubscript{i}, the action of sphingosine 1-phosphate (S1P) is novel. In these cells, S1P, a biologically active lysophospholipid, greatly enhances increases in intracellular cAMP triggered by the ligands for G\textsubscript{i}-coupled receptors while having only a minimal effect by itself. The enhancement of cAMP by S1P is resistant to pertussis toxin and independent of intracellular calcium. Studies with RNAi and chemical perturbations demonstrate that the effect of S1P is mediated by the S1P\textsubscript{2} receptor and the heterotrimeric G_{13} protein. Thus in these macrophage cells, all four major classes of G proteins can regulate intracellular cAMP.

Cyclic adenosine 3',5'-monophosphate (cAMP), a ubiquitous second messenger, mediates a wide range of cellular functions including cell metabolism (1), cell proliferation and differentiation (1), immune responses (2, 3), memory formation (4), and cardiac contractility (5). Canonically, the concentration of intracellular cAMP is regulated by two distinct families of enzymes. The transmembrane adenylyl cyclases (ACs)\textsuperscript{3} synthesize cAMP from adenosine triphosphate (6, 7), whereas the cAMP-specific phosphodiesterases metabolize cAMP to biologically inactive adenosine 5'-monophosphate (8, 9). ACs are primarily activated by G\textsubscript{\alpha}\textsubscript{q}, but their activities can also be differentially regulated by G\textsubscript{\alpha}\textsubscript{q}, G\textsubscript{\beta}\textsubscript{\gamma}, or Ca\textsuperscript{2+} (10, 11). The activities of various phosphodiesterases can be regulated by protein kinase A (PKA), extracellular-regulated kinase (ERK), phosphoinositide 3-kinase, and the concentration of cAMP itself (12–16). Thus integration of signaling by stimuli that can regulate the intracellular concentration of cAMP will depend strongly on the various pathways and the subtypes of ACs and phosphodiesterases expressed in individual cells at any given time.

Assessment of the regulation of intracellular cAMP in vivo has only become possible recently. Zaccolo \textit{et al.} (17) first described a FRET sensor for cAMP based on the cAMP binding domain of PKA. Subsequently, several reports have described FRET sensors for cAMP based on binding of the nucleotide to the Epac proteins (18–21). While these FRET sensors have been effective for measuring changes and localization of cAMP in single cells, measurements are tedious. Furthermore, the requirement for excitation of donor molecules produces a range of problems including cell damage, photobleaching, and low signal-to-noise ratios due to intrinsic cellular autofluorescence. This precludes use of the FRET sensors in high throughput population assays. In contrast, BRET (bioluminescence resonance energy transfer) sensors use an enzymatic reaction to produce energy emission in the donor, usually produce better signal-to-noise ratios, and are better suited for high throughput population assays (22). We report here the development and characterization of an Epac-based BRET sensor for cAMP (CAMYEL) with improved dynamic range and a method to quantify intracellular cAMP changes.

This sensor was further used to characterize a novel phenomenon identified in RAW 264.7 cells. The Alliance for Cellular Signaling (AfCS) has conducted a comprehensive double ligand screen using the mouse macrophage-like cell line, RAW

\textsuperscript{5}This work was supported by National Institutes of Health Grant GM 62114, the Robert A. Welch foundation (to P. C. S.), and the Alfred and Mabel Gilman Chair in Molecular Pharmacology (to P. C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{6}The on-line version of this article (available at http://www.jbc.org) contains supplemental data and Figs. S1–S7.

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3 The abbreviations used are: AC, adenylyl cyclase; ISO, isoproterenol; PGE, prostaglandin E\textsubscript{2}; C5a, complement C5a; S1P, sphingosine 1-phosphate; CAMYEL, cAMP sensor using YFP-Epac-RLuc; EIA, enzyme-linked immunoassay; TER, terbutaline; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; RL, Renilla luciferase; SERCA, sarcoplasmic reticulum calcium ATPase; ERK, extracellular signal-regulated kinase; PKA, cAMP-dependent protein kinase.
264.7, to examine the extent of ligand interactions on a variety of downstream outputs, including cAMP (23). One of the striking results is the interaction between the lysophospholipid, sphingosine 1-phosphate (SIP), and ligands for receptors that stimulate cAMP. SIP alone does not significantly elevate either cAMP or Ca^2+ in RAW 264.7 cells. However, SIP greatly increases the amount of intracellular cAMP stimulated by iso-proterenol (ISO) and prostaglandin E2 (PGE). Other ligands that stimulate mobilization of Ca^2+ in RAW 264.7 cells, such as complement C5a and uridine 5’-diphosphate (UDP), also enhance stimulation of cAMP by ISO and PGE, but are less efficacious than SIP.

Receptors for SIP, formerly known as endothelial differentiation genes (Edg), are heptahelical transmembrane receptors that can variously interact with at least three G protein subfamilies, Goi, Goq, and Go12/13 (25–27), to affect regulation of phospholipase C (Gq, and G12), phosphoinositide 3-kinase (Gq), and guanine nucleotide exchange factors for Rh (Go12/13) (28–33). SIP has also been shown to regulate inhibition of intracellular cAMP by a receptor-dependent Goi-mediated mechanism (29, 31, 32). Although SIP has been shown to stimulate intracellular cAMP in cells overexpressing SIP receptors, there is no evidence that the SIP receptors couple directly to Goi and the mechanism remains unclear (30).

Using the CAMYEL sensor, we identified roles for SIP2 receptors and G13 proteins in mediating the synergistic effect of SIP on cAMP responses. This is the first evidence that a G13 pathway is involved in the regulation of cAMP. Kinetic experiments indicate that the effect of SIP has rapid onset and works through increasing the rate of synthesis of the cyclic nucleotide.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Isoproterenol, prostaglandin E2, complement C5a, uridine 5’-diphosphate, 8-bromo cAMP, and phosphodiesterase inhibitors (Sigma), sphingosine 1-phosphate (Avanti Polar Lipids), terbutaline (Sigma), ICI 118551 (Biomol Research), SEW2871 (Cayman Chemical), and JTE 013 (Tocris Bioscience) were purchased from the sources indicated.

**DNA Constructs**—Epac1 (amino acids 148–881) was generated by RT-PCR with human brain RNA as template using the primer pair 5’-CTC CGC GGA CCC GAG CCC GTG GGA ACT C-3’ and 5’-GTG AAT TCT GGC TCC AGC TCT CGG GAG AG-3’. Two point mutations, T781A and F782A, which eliminate the guanine nucleotide exchange activity of Epac (21), were created using site-directed mutagenesis with the QuikChange kit from Stratagene. Citrine with the monomeric mutation A206K and Renilla luciferase (RL) were amplified by PCR using primer pairs: 5’-ATG GAT CCA TGG TGA GCA AGG GGC AG-3’ and 5’-CGG CGG AGC TTG TAC AGC TGG TCC ATG ATG-3’; 5’-GAA TTC TCC AGT TCC AAG GTG TAC G-3’ and 5’-GCC GGC GCC TGC TAC TCG TCC TTC TCC AGC-3’. Fragments were then fused to the N and C termini of Epac1 as shown in Fig. 1. Circularly permuted citrine was generated by sewing PCR; constructs were equivalent to those described for the improved YFP, Venus (34). pcDNA3.1-His from Invitrogen was used for expression in mammalian cells; pQE30 from Qiagen was used for expression in bacteria. The pFB-neo vector from Stratagene was used to deliver the cAMP sensor into RAW 264.7 cells via retroviral infection.

**Cell Culture and Transfection**—Protocols for culturing RAW 264.7 cells and for transfection of DNA and siRNA or retroviral infection can be found at the AICS website. Briefly, RAW 264.7 cells (obtained from ATCC) and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 20 mM NaHEPES, pH 7.4. Transfection with DNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Retrovirus was made with the Phoenix Amphotropic packaging cell line (Orbigen). Infection of RAW cells was initiated by applying virus-containing supernatant harvested from the packaging cell line with 6 μg/ml polybrene on top of the cells and spinning at 1,200 × g at 32 °C for 2 h. Cells were then cultured with the viral supernatant for 1 day at 32 °C followed by removal of the viral medium and further culturing with fresh medium containing 500 μg/ml G418 at 37 °C.

**Gene Knockdown by RNAi**—siRNA pools of oligomers targeting SIP1, SIP2, and G13 were SMARTPool products purchased from Dharmacon. The sequence for the SIP2-B oligomer (5’-GCA TGT CAC TCT GTC CTG A-3’) is unique from those in the SMARTPool. RAW cells were transfected with 400 nM siRNA using Lipofectamine 2000. Cells were plated into 96-well tissue culture plates at 24 h post-transfection and assayed at 48 h post-transfection. Samples were taken for Western blot or qRT-PCR to assess the knockdown level of protein or mRNA, respectively. Cells were lysed and blotted with antisemur B-860 to detect Goi as described (35). Respective primers for qRT-PCR of the SIP1 and SIP2 receptors are: (F) 5’-CCG TGT AGA CCC AGA GCC TGT GTG AAT-3’ and (R) 5’-TTT TAT TGG GCT GGT CCT TGT-3’; (F) 5’-AGC CAA CAG TCT CCA AAA CCA-3’ and (R) 5’-GGG CTC AGC ACT GGC TAG G-3’. The qRT-PCR reactions were done with an ABI 7500 Real-Time PCR system from Applied Biosystems.

**EIA Assay for cAMP**—Cells were plated on 96-well tissue culture plates 1 day prior to the treatment with ligands. Prior to treatments, cells were cultured in serum-free medium for 1 h. Ligands were then added to stimulate the cells. After the addition of ligands, reactions were stopped at the indicated times by removal of medium and cell lysis with 65% ethanol. Cell lysates were then dried and assayed using the cAMP Biotrak EIA kit (Amersham Biosciences).

**Measurement of Fluorescence in Vitro**—Cells that express the CAMYEL sensor were lysed with buffer containing 20 mM NaHEPES, pH 7.4, 50 mM KCl, 50 mM NaCl, 2.5 mM MgCl2, 0.2% Nonidet P-40, 5 mM dithiothreitol, and a mixture of protease inhibitors (Roche Applied Science). Bacterial expressed protein with an N-terminal His tag was purified with Ni-NTA resin. Coelenterazine-h (2 μM final concentration) was added to the cell lysates or purified proteins immediately prior to measurements of fluorescence emission spectra using a Spectrofluorometer MD-5020 (Photon Technology International).

**Assay of BRET in Live Cells**—Cells were plated in 96-well solid white tissue culture plates (Greiner) at a density of 60,000 cells per well the day before assays. Cells were serum-starved in Hank’s balanced salt solution, pH 7.4, for 1 h before treatments. The BRET assay was carried out with a POLARstar Optima.
plate reader from BMG LabTech. Emission signals from RL and YFP were measured simultaneously using a BRET\(^1\) filter set (475–30/535–30). Cells in each well were assayed in 80 \(\mu\)l of Hank’s balanced salt solution with 2 \(\mu\)M coelenterazine-h, and stimulations were initiated by injection of 20 \(\mu\)l of 5\(\times\) ligand.

**Calculation of cAMP Concentration**—Ratiometric data were converted to cAMP concentration using Equation 1.

\[
[cAMP] = K_d \times \left( \frac{(R - R_{\text{max}})}{(R_{\text{max}} - R)} \right) 
\]  

*(Eq. 1)*

The \(K_d\) and Hill coefficient for association of cAMP with the sensor were determined to be 8.8 \(\mu\)M and 1, respectively. \(R\) is the intensity ratio of RL to YFP. \(R_{\text{max}}\) is obtained by adding 2 \(\mu\)M 8-bromo cAMP to the cells at the end of the assay. \(R_{\text{min}}\) is arbitrarily set to be the average ratio of measurements with unstimulated cells minus 4 S.D. (see supplementary data for details).

**RESULTS**

**An Epac-based BRET Sensor for cAMP-CAMYEL**—Several FRET sensors for detection of cAMP have been described in recent articles (19–21). Because the sensor containing an inactive cytosolic mutant form of human Epac-1 appeared to have the best signal-to-noise ratio (21), it was used as a basis for development of a BRET sensor. The design of the sensor is shown in Fig. 1A; it utilizes the enhanced variant of YFP, citrine (36), and *Renilla* luciferase as the BRET pair with human Epac1 inserted in between. Spectra of the expressed sensor protein revealed strong resonance energy transfer that was significantly reduced in response to cAMP, as expected from the FRET sensors and known structural changes in the molecule (21, 37, 38).

We sought to further improve the signal by replacing citrine with circularly permuted versions of the protein. Such circular permutations of the fluorescent proteins have been shown to vastly improve the FRET signal in a sensor for calcium (34). This improvement in FRET efficiency may be due to altered fluorophore orientation of the permuted proteins, and we reasoned that similar changes would replace to the efficiency of BRET sensors. Of the five different circular permutations screened, we found that replacement of citrine with citrine-cp229 improved the changes of BRET ratio upon binding cAMP by 2-fold (Fig. 1). The improved sensor gives an increase in BRET ratio (RLuc/YFP) of about 70% with cAMP and was named CAMYEL (cAMP sensor using YFP-Epac-RLuc).

The CAMYEL protein was expressed in bacteria and purified for further characterization. A concentration response analysis with cAMP indicated a \(K_d\) and \(n\) (Hill coefficient) for binding of 8.8 \(\mu\)M and 1, respectively (supplemental Fig. S1); these are similar to binding properties reported for the FRET sensor (21). While the activity of luciferase can be influenced by calcium, resonance energy transfer should be independent of these changes in absolute signals if calcium does not cause any conformational changes in the sensor protein. This appears to be the case because the BRET ratios, either in the presence or absence of cAMP, were not influenced by concentrations of calcium up to 1 \(\text{mM}\) (supplemental Fig. S2). Additional experiments indicated that the BRET signals were also independent of changes in pH within the tested range (pH 6.5–8.0, supplemental Fig. S2). The response time of the sensor was assessed by measured changes in BRET when the sensor was exposed to abrupt increases or decreases in cAMP. Such changes depend on both the association and dissociation of cAMP and the ensuing changes in conformation. *In vitro*, the change of BRET ratio in response to altered cAMP concentrations appears to be complete within 1 to 2 \(\text{s}\) (supplemental Fig. S3). This rate of signal change is well suited for measuring the kinetics of change in intracellular concentrations of cAMP.

**Regulation of Cytosolic cAMP in RAW 264.7 Cells**—One of the outputs measured in a survey by the Alliance for Cellular Signaling for the complexity of interactions among signaling pathways was the regulation of the classical second messenger, cAMP. A traditional enzyme-linked immunoassay (ELA) for total intracellular cAMP was used to identify two ligands, isoproterenol (ISO) and prostaglandin E\(_2\) (PGE), which effectively increased this second messenger in RAW cells. These two ligands were subjected to a comprehensive double ligand screen with over twenty additional ligands to look for non-additive interactions (23). Ligands that elicit calcium responses in RAW cells, such as complement C5a and uridine 5’-diphosphate (UDP), enhanced cAMP responses triggered by ISO or PGE. Interestingly, S1P, which at best induced only minimal PGE. Interestingly, S1P, which at best induced only minimal

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**FIGURE 1. An Epac-based BRET sensor for cAMP (CAMYEL).** A, a schematic drawing of the domain structure of CAMYEL. The inactive cytosolic mutant form of human Epac-1 (amino acids 149–881, T781A, F782A) (21) was flanked by citrine or a circularly permuted citrine-cp229 and *Renilla* luciferase (RL). B and C, comparison of emission spectra of Citrine-Epac-RL (B) and Citrine-cp229-Epac-RL (C) in the presence and absence of 100 \(\mu\)M cAMP. HEK293 cells were transiently transfected with either construct. Cells were lysed with buffer containing 20 mM NaHEPES, pH 7.4, 50 mM KCl, 50 mM NaCl, 2.5 mM MgCl\(_2\), 0.2% Nonidet P-40, 5 mM dithiothreitol, and protease inhibitors. Emission spectra were measured in the presence of 2 \(\mu\)M coelenterazine-h substrate with or without 100 \(\mu\)M cAMP.
produced normal calcium and cAMP (as measured by EIA) responses to an array of ligands. In such cell lines the BRET ratio, RLuc/YFP, can then be used to continuously monitor intracellular cAMP in real time (Fig. 2B). The dynamic range of the sensor in RAW cells was measured using 2 mM 8-bromo cAMP, a cell-permeable analog of cAMP, to saturate the sensor in vivo.

Stimulation of β-adrenergic receptors with ISO led to rapid decreases of the BRET signal hence increases of the fluorescence intensity ratio, RLuc/YFP (Fig. 2B). The response peaked at ~20–30 s after addition of ligand and declined to a sustained level. This response profile is qualitatively the same as measured by EIA. The BRET sensor also recapitulates the effect of S1P and UDP on production of cAMP. Addition of either S1P or UDP alone elicited minimal cAMP responses (Fig. 2B), although UDP stimulates robust increases in intracellular calcium in RAW cells. This confirms in vitro results that calcium does not affect the BRET ratio of the sensor. Simultaneous addition of either S1P or UDP together with ISO led to greater changes in BRET at the peak of the response and subsequent decreases over time to a sustained level that was equivalent to that obtained with the addition of ISO alone.

To better assess the dynamics of changes in intracellular cAMP, we converted the ratiometric results shown in Fig. 2B to concentrations of cAMP (Fig. 2C, see “Experimental Procedures” and supplementary information). Using this method of conversion, we can then compare quantitative changes in cAMP measured with the BRET sensor to those obtained with the EIA. As shown in Fig. 2C, S1P or UDP synergistically increases the peak cAMP response induced by ISO alone by 2–4 or 1.5–2 folds, respectively. This slightly higher ratio of enhancement than that observed with EIA is likely due to two differences in the assessments. First, the peak response is usually missed by the EIA. Second, the sensor measures free intracellular cAMP, whereas the EIA also measures bound nucleotide that will be proportionally higher at lower concentrations.

Enhancement of cAMP by S1P Is Caused by Increased Synthesis of cAMP—Use of the CAMYEL sensor allows us to dissect the effect of S1P on cAMP through detailed kinetic measurements. The intracellular concentration of cAMP is determined by a dynamic balance between its biosynthesis via adenylyl cyclases and its degradation by phosphodiesterases. The enhancement observed with S1P could be due to either stimulation of synthesis or inhibition of degradation. We first compared rates of cAMP increases when cells were stimulated by either ISO alone or combination of ISO and S1P. The synergistic effect of S1P is observed at the earliest times after ligand addition with a greater than 4-fold increase in rate throughout the approach to peak values (Fig. 3, filled symbols). This suggests that the enhancement by S1P is temporally well-coupled to the early processes causing activation of ACs.

Inhibitors of phosphodiesterases were then used to reduce degradation of cAMP. Under these conditions, ISO produced a much greater rise in intracellular cAMP, which was readily sustained over longer times. However, blocking degradation of cAMP did not reduce the enhanced rate or the extent of cAMP accumulation affected by S1P (Fig. 3, open symbols). Similar results were obtained with PGE, and its combination with S1P (data not shown). These results clearly indicate that modulation of phosphodiesterase activity is not a primary mechanism by which S1P enhances cAMP.
Stimulation of cAMP by S1P Is Related to the Activities of the Gs Pathway—When RAW cells expressing the CAMYEL sensor are treated with ISO, the cells rapidly increase the level of free intracellular cAMP to a peak response (≈20–30 s); this is usually followed by a rapid decrease to a sustained response that declines slowly over time. To examine the temporal coupling between the effect of S1P and activation of the Gs pathway, S1P was added either simultaneously with ISO or during the sustained phase of the ISO response (Fig. 4A). Interestingly, the synergistic effect of S1P on intracellular cAMP was observed throughout the experiment. Moreover, the increase in cAMP induced by addition of S1P is directly related to the concentration of intracellular cAMP stimulated by ISO at the time of S1P addition (Fig. 4B). Thus, the relative enhancement by S1P, a maximal increase of about 300% over the stimulation produced by ISO alone, remained the same regardless of when the ligand was added after ISO. This indicates that not only can S1P exert its effect as long as the synthetic pathway for cAMP is active, but also the mechanism for the effect of S1P is not altered by the magnitude of cAMP synthesis activated by GPCR-Gs pathways.

The dependence of the S1P effect on the synthetic pathway of cAMP was further tested by termination of stimulation of the /H9252-adrenergic receptor with a specific antagonist, ICI-118551. For this study, the agonist terbutaline (TER), which has a lower affinity and faster rate of dissociation than ISO, was used. As shown in Fig. 4C, S1P enhanced the cAMP response triggered by TER as it did to that of ISO. Addition of ICI-118551 led to a rapid decline of intracellular cAMP that had been stimulated by TER (red trace, open circles). If the addition of ICI-118551 preceded that of S1P by 12 s, the effect of S1P was abolished (green trace, inverted triangles). Therefore the S1P effect requires activation of the Go, pathway and adenylyl cyclase. Because the adenylyl cyclase activity in the RAW cells is unresponsive to forskolin, a simple requirement for active adenylyl cyclase could not be tested.

Enhancement of cAMP by UDP and C5a Is Mediated by Calcium and Gi Pathways, Respectively—The activity of adenylyl cyclases can be regulated by calcium and the Gi pathway (11). To examine the role of calcium in the enhancements of cAMP by UDP, C5a, and S1P, intracellular and extracellular calcium
were depleted by addition of the SERCA inhibitor thapsigargin and calcium chelator EGTA. The synergetic effects of S1P, UDP or C5a on cAMP responses induced by ISO were assessed with a sequential ligand format where addition of the enhancing ligands triggers a second peak response after the initial response of cAMP to ISO (Fig. 5). Attenuation of intracellular calcium did not affect the first cAMP response peak induced by ISO, nor did it significantly affect the second peak response triggered by S1P or C5a (Fig. 5, A and C). However, the enhancement by UDP was largely ablated without intracellular calcium (Fig. 5B).

Inactivation of the G\textsubscript{i} pathway was achieved by treatment of the cells with pertussis toxin. As shown in Fig. 5, inactivation of the G\textsubscript{i} pathway effectively ablated the effect of C5a on cAMP without significantly affecting the cAMP response of ISO alone (Fig. 5C) or the synergistic effects of UDP and S1P (Fig. 5, A and B). In contrast to UDP and C5a, the stimulation of cAMP observed with S1P could not be explained by these known mechanisms.

The S1P\textsubscript{2} Receptor Mediates the Changes in cAMP Caused by S1P—RAW 264.7 cells express two receptors for sphingosine 1-phosphate, S1P\textsubscript{1} (Edg1) and S1P\textsubscript{2} (Edg5), as determined by RT-PCR. A knockdown approach with RNAi was used to determine which receptor is responsible for mediating the effect of S1P. Pools of four different siRNA oligomers specifically targeting either S1P\textsubscript{1} or S1P\textsubscript{2} were transfected into RAW cells carrying the CAMYEL sensor. The effects of gene specific knockdown were assessed by qRT-PCR (Fig. 6A). Knockdown of the S1P\textsubscript{2} receptor greatly reduced the synergistic effect of S1P without changing the cAMP response triggered by ISO alone. This phenomenon is evident with either simultaneous addition of ISO and S1P (Fig. 6C) or sequential addition of ISO followed by S1P (Fig. 6D). A single siRNA oligon (S1P\textsubscript{2}-B) that targets S1P\textsubscript{2} was selected independently of the oligomer pool and used to confirm the specificity of the S1P\textsubscript{2} knockdown phenotype (Fig. 6D). A partial knockdown of S1P\textsubscript{1} appeared to slightly enhance the synergistic response of cAMP to S1P. However, the enhancing effect appeared to be nonspecific as an increase was also observed when the cells were stimulated with the combination of ISO and UDP (Fig. 6B).

The receptor knockdown result was further confirmed by drug perturbations. SEW2871 is an agonist for the S1P\textsubscript{1} receptor (39). Addition of SEW2871 following that of ISO failed to reproduce the effect of S1P (Fig. 6E). However, addition of JTE-013, an antagonist for the S1P\textsubscript{2} receptor (40), at 12 s prior to S1P addition greatly reduced the effect of S1P on the cAMP response triggered by ISO (Fig. 6F). Together these results indicate that S1P preferentially uses the S1P\textsubscript{2} receptor to enhance intracellular concentrations of cAMP in RAW cells.

Stimulation of cAMP by S1P Is Mediated by G\textsubscript{13}—Whereas S1P\textsubscript{1} is reported to couple primarily to G\textsubscript{i}, S1P\textsubscript{2} can act through G\textsubscript{q}, G\textsubscript{13}, and G\textsubscript{12/13} to stimulate a variety of pathways (30, 31, 33). Because inactivation of the G\textsubscript{i} pathway did not attenuate the S1P effect on cAMP and the lack of calcium response to S1P indicates that activation of G\textsubscript{q} is unlikely, we explored the potential role of G\textsubscript{13} in the S1P synergism. As shown in Fig. 7, transfection of cells expressing the CAMYEL sensor with siRNAs targeting G\textsubscript{13} greatly reduced the enhancement of S1P on the cAMP response generated by ISO (Fig. 7, C and D). In contrast, the response of cAMP to ISO alone or its enhancement by UDP, a ligand which likely acts through a G\textsubscript{q}-coupled mechanism, remained unaffected (Fig. 7B). The enhanced effect of S1P on the cAMP response of PGE\textsubscript{2} was also attenuated by the knockdown of G\textsubscript{13} (Fig. 7E).

The specificity of G\textsubscript{13} involvement in the S1P synergy on cAMP was assayed by using a microRNA-based shRNA delivered via retroviral infection to knockdown G\textsubscript{13}. The targeting sequence was selected independently of the pooled oligomers, and a similar phenotype was observed when cAMP was measured either with the CAMYEL sensor or an EIA (supplemental Fig. S5). The partial reduction of the response is consistent with an incomplete knockdown of the G\textsubscript{13} but could also be indic-
ative of contributions from a second pathway. Attempts to assess potential involvement of the most obvious candidate, $G_{\alpha 13}$, have been hampered by our inability to produce effective knockdown of this $G_{\alpha 13}$ subunit in RAW cells.

FIGURE 6. The S1P$_2$ receptor transducessignaling by S1P for modulation of intracellular cAMP. A, RAW cells expressing CAMYEL were transiently transfected with control siRNA or pools of four siRNA oligomers (designated as –) targeting either the S1P$_1$ or S1P$_2$ receptors. $B$–$D$, cells were assayed for cAMP responses to simultaneous addition of 16 nM ISO and 0.5 $\mu$M UDP ($B$), simultaneous addition of 16 nM ISO and 10 nM S1P ($C$), and sequential addition of 16 nM ISO followed by 10 nM S1P ($D$). Knockdown of S1P$_2$ using a single oligomer (S1P$_2$-B) that is independent of the pool of four oligomers resulted in a similar phenotype ($D$).

FIGURE 7. $G_{\alpha 13}$ is required for the synergy of S1P on Gs-mediated cAMP responses. A, RAW cells expressing CAMYEL were transiently transfected with control siRNA or a pool of four siRNA oligomers (designated as –) specifically targeting $G_{\alpha 13}$. The presence of $G_{\alpha 13}$ was assessed by Western blot. The reduction of the $\alpha$-subunit in cells treated with four siRNA oligomers targeting $G_{\alpha 13}$ was about 60%. $B$–$D$, response of cAMP in control transfected and $G_{\alpha 13}$ knockdown cells to simultaneous addition of 16 nM ISO and 0.5 $\mu$M UDP ($B$), simultaneous addition of 16 nM ISO and 10 nM S1P ($C$), and sequential addition of 16 nM ISO followed by 10 nM S1P ($D$). $E$, synergy of S1P on intracellular cAMP induced by PGE is also reduced in $G_{\alpha 13}$ knockdown cells. Ligands were added at time 0 or as indicated. Error bars represent the standard deviation of results from four experiments of 2 independent transfections. Errors were similar for the other traces but left out for clarity.
DISCUSSION

We successfully converted an Epac-based FRET sensor for cAMP to a BRET sensor. The signal of the new sensor was improved 2-fold by using circularly permuted fluorescent proteins, a technological advance used previously to alter the efficiency of resonance energy transfer between FRET pairs (34). The use of circularly permuted citrine in CAMYEL demonstrates the utility of this method for evolving better sensors using bioluminescent donors as well. With its good signal-to-noise ratio and excellent dynamic range, CAMYEL is ideal for continuous in vivo monitoring of cAMP metabolism in populations of cells.

The use of permeable cAMP analogs to saturate output further allows calibration of the sensor and conversion of ratio-metric measurements to real estimates of cAMP concentration. Its rapid and reversible response makes the sensor an excellent tool to measure the kinetics of this signaling pathway and hence support molecular modeling of this signaling system, one of the goals of the Alliance for Cellular Signaling. The use of CAMYEL for exploring regulation of signaling pathways is described herein. The sensor also has the potential to support high throughput genome-wide genetic screens, such as an RNAi screen, to identify new players involved in the regulation of cAMP as well as screens for small molecule drugs.

The BRET sensor was used to characterize the cAMP responses to dual ligands in mouse macrophage-like RAW 264.7 cells. Three ligands, which do not stimulate cAMP by themselves, were found to enhance production of cAMP induced by G_α_5-coupled receptors via three distinct mechanisms. The effects of UDP and C_5α on cAMP responses are likely due to canonical regulation of adenylyl cyclase activity via calcium regulated by G_α_5 proteins and the G_i pathway, respectively. Interestingly, the ability of S1P to enhance cAMP production stimulated by G_α_5-coupled ligands uses neither of these known pathways. Rather, S1P acts through S1P_2 receptors and uses the heterotrimeric G_13 proteins to couple to the synthesis of cAMP. This is the first evidence that a member of the G_12/13 subclass of G proteins can robustly regulate production of this classic and ubiquitous second messenger.

One remaining issue is to identify the site of action for stimulation of cAMP synthesis by the S1P/G_13 pathway. Three possible sites of action are the stimulatory receptors, G_α_5, or direct action on adenylyl cyclases. Enhancement of the action of β-adrenergic receptors on G_α_5, perhaps facilitated by oligomerization of receptors or modification of receptors, would likely be accompanied by a shift in the concentration response curve of ISO to a lower E_C_{50} in the presence of S1P. However, the E_C_{50} of the ISO response remained the same, regardless of S1P (supplemental Fig. S4). In addition, the S1P/G_13 pathway also synergistically stimulates cAMP triggered by PGE. This would require the S1P/G_13 pathway to somehow couple with both the β-adrenergic and prostaglandin receptors. Enhancement of the activity of G_α_5 by the S1P/G_13 pathway would require pre-activation of G_α_5 as S1P by itself does not activate G_α_5. Examples of such regulation are not known but mechanisms might include inhibition of the GTPase activity of G_α_5 in analogy to the action of cholera toxin. This seems unlikely because the G_α_5 pathway is common to all cells and this enhancement of cAMP synthesis by S1P/G_13 is not observed in many other cells (such as HEK293 and HeLa) where S1P activates G_13 (data not shown). The most likely target is one or more subtypes of adenylyl cyclase, because specific subtypes have already been described that account for the impact of calcium and G_13. RAW 264.7 cells minimally express four adenylyl cyclases, AC2, AC3, AC7, and AC9, where AC2 could account for the G_13-dependent effects of C5a via a βγ mechanism and AC3 could respond to stimulation via calcium/calmodulin. We are in the process of determining the role of each of these cyclase subtypes in the cAMP response of RAW cells to hormonal stimuli.

A hint to the mechanism of S1P synergy may be evident in the brief but significant increase in cAMP when S1P and the β-adrenergic antagonist were added to cells stimulated with TER (Fig. 4C). In the absence of S1P, the antagonist initiates an almost immediate decline in cAMP, suggesting rapid dissociation of TER from the receptor and coincident inactivation of the receptor and adenylyl cyclase. When S1P was added simultaneously with the antagonist, there was an immediate increase in cAMP that lasted about 10 s before the second messenger started to decline. This indicates that adenylyl cyclase was not rapidly inactivated under this condition and suggests S1P can act rapidly by some mechanism to increase the lifetime of activated adenylyl cyclase, either by itself or in complex with G_α_5.

How does the G_13 pathway regulate cAMP synthesis? Conceivably, release of the βγ subunits from G_13 could account for the enhancing activity of S1P in a fashion similar to that of βγ subunits released from G_i to regulate the activity of ACs (10, 11, 41, 42). Alternatively, G_α_13 could act directly on adenylyl cyclase. No evidence for this has yet been generated in attempts to reconstitute the phenomena with cellular membranes and activated G_α_13 proteins (data not shown). A well-characterized pathway stimulated by G_13 is Rho-dependent regulation of cytoskeleton and gene transcription (43, 44). We used inhibitors of Rho and Rho kinases to block this pathway but failed to disrupt the S1P synergy (supplemental Fig. S6). At this time, further elucidation of mechanism will likely require identification of additional players in the pathway. Use of the CAMYEL sensor makes it possible to screen for such a player in a genomewide high throughput fashion.

What is the physiological relevance of this S1P/G_13 effect in intracellular cAMP regulation? To begin answering this question, we examined the cAMP responses in primary macrophages derived from mouse bone marrow. The application of S1P robustly enhanced the stimulation of cAMP production observed with agonists of G_α_5-coupled receptors and its effect appears to be prolonged into the sustained phase of the cAMP response (supplemental Fig. S7). Interestingly, a similar phenomenon of enhancement on cAMP responses has been reported for thrombin in a human erythroleukemia cell line and human erythroid progenitor cells (45–47). In both cell lines, thrombin does not trigger either cAMP or calcium responses on its own, yet it can potentiate G_α_5-activated increases in intracellular CAMP. A mechanism for this effect of thrombin was not reported. Regulation of cAMP plays an important role in cell growth, differentiation, and cellular functions in the immune system. Both S1P and thrombin are also known to modulate...
immune responses in various hematopoietic cells (24, 26). Conceivably, the ability of other GPCR pathways to enhance intracellular cAMP responses may help reinforce certain cellular responses or lead to concerted cellular behaviors. It will be of interest to determine the downstream targets of enhanced cAMP responses and their effect on cellular function and immune function in animals. For example, a major function of macrophage cells is to produce and release cytokines upon activation; these processes are strongly modulated by cAMP in the RAW macrophages and currently under investigation by the AfCS.

Acknowledgments—We thank Jana Hadas for help with protein purification and Drs. William Singer and Scott Gibson for plasmids.

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