3 High-Throughput FRET Assays for Fast Time-Dependent Detection of Cyclic AMP in Pancreatic β Cells

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CONTENTS

3.1 INTRODUCTION

Pancreatic β cells located in the islets of Langerhans secrete insulin in response to the increase of blood glucose concentration that occurs after a meal.[1] Insulin secreted in this manner acts as a circulating hormone to suppress hepatic glucose

production while also promoting glucose uptake into striated muscle and fat.[2] When this process of systemic glucose homeostasis is disrupted, chronic hyperglycemia ensues, as is the case for patients diagnosed with type 2 diabetes mellitus (T2DM).[3] Current drug discovery efforts for the treatment of T2DM seek to identify new agents that will lower the levels of blood glucose by stimulating insulin secretion.[4] Here, we summarize efforts to achieve this goal, with emphasis on the identification of adenosine-3′,5′-cyclic monophosphate (cAMP) elevating agents that directly stimulate insulin release from β cells.[5] We also describe a new fluorescence resonance energy transfer (FRET) assay with which to implement a high-throughput screen of β cell cAMP-elevating agents. This assay uses cell lines transfected with genetically encoded cAMP biosensors that allow the detection of cAMP in a fast and time-dependent manner.

3.2 cAMP SIGNALING IN PANCREATIC β CELLS

Glucose is the primary stimulus for insulin secretion from pancreatic β cells,[6] and this action of glucose is potentiated by cAMP-elevating agents that activate G protein–coupled receptors (GPCRs) expressed on β cells (Figure 3.1).[7] Two such cAMP-elevating agents are exenatide (Byetta) and liraglutide (Victoza), both of which are prescribed

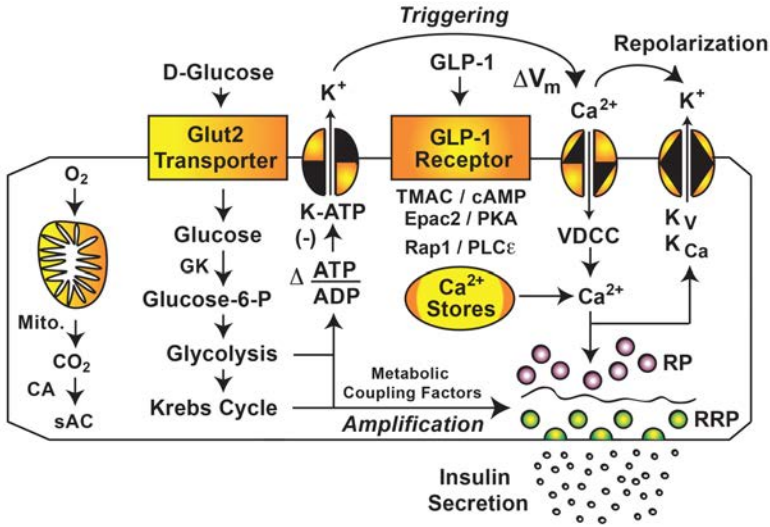

FIGURE 3.1 Glucose metabolism in β cells stimulates depolarization (ΔV_m) and entry of Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCCs). Ca^{2+} triggers the exocytosis of insulin from secretory granules located in a readily releasable pool (RRP) and a reserve pool (RP). A cortical actin barrier (wavy line) separates the RRP and RP, and it must undergo *remodeling* for glucose to stimulate exocytosis of the RP. GLP-1 activates its GPCR to potentiate glucose-stimulated insulin secretion in a PKA- and Epac2-mediated manner. Downstream effectors of Epac2 include Rap1 GTPase and a Rap1-regulated phospholipase C-epsilon (PLCε). Membrane repolarization occurs in response to the activation of voltage-dependent K^+ channels (K_V) and Ca^{2+}-activated K^+ channels (K_{Ca}). Mitochondrial (Mito.) respiration generates CO_2 that is converted by CA to generate HCO_3^- that activates sAC.

as blood glucose–lowering agents for the treatment of T2DM.[8] Exenatide and liraglutide are insulin secretagogues with blood glucose–lowering properties because they activate a GPCR that normally binds the cAMP-elevating hormone glucagon-like peptide-1 (GLP-1) in β cells.[9] GLP-1 is an insulinotropic peptide released from intestinal L-cells in response to the ingestion of a meal, and it complements the action of intestinally absorbed glucose to stimulate pancreatic insulin secretion.[10]

Binding of GLP-1 to the GLP-1 receptor (GLP-1R) activates heterotrimeric G_s proteins, thereby stimulating the activities of transmembrane adenylyl cyclases (TMACs) in β cells (Figure 3.1).[11] TMACs catalyze the synthesis of cAMP, a cytosolic second messenger that activates two distinct classes of cAMP-binding proteins: (1) protein kinase A (PKA, a serine/threonine protein kinase), and (2) cAMP-regulated guanine nucleotide exchange factors designated as Epac1 and Epac2.[12–15] Evidence exists that PKA and Epac2 mediate the actions of GLP-1R agonists to increase the efficacy and potency of glucose as a stimulus for insulin secretion.[12–15] Furthermore, PKA and Epac2 seem to mediate an action of GLP-1R agonists to repair defective stimulus-secretion coupling in the β cells of patients with T2DM.[12–15] Thus, GLP-1R agonists are not simply β cell glucose sensitizers, but they also induce β cell *glucose competence* under conditions of metabolic stress.[16–21] Potentially, the PKA and Epac2 signaling mechanisms in β cells can be targeted to identify new pharmacological agents for use in the treatment of T2DM.

Because β cells express a surprisingly diverse assortment of GPCRs,[22] there is good reason to expect that new classes of insulin secretagogues will be identifiable in high-throughput screens that are based on the ability of a candidate compound to stimulate cAMP production in a GPCR-mediated manner. Likely GPCRs that can be targeted in β cells are the cAMP-elevating GPCRs for fatty acid amides (GPR119), bile acids (TGR5), L-arginine (GPRC6A), pituitary adenylyl cyclase–activating polypeptide (PACAP-R), glucose-dependent insulinotropic peptide (GIP-R), and glucagon (Gluc-R).[4,7,22] Because β cells also express GPCRs that inhibit cAMP production,[23] it might be possible to identify antagonists of GPCRs that indirectly increase levels of cAMP to stimulate insulin secretion. Once identified, the properties of all such cAMP-elevating agents can be evaluated in secondary screens of glucose-stimulated insulin secretion (GSIS) using isolated islets,[24–26] or in single-cell assays of β cell ion channel function, Ca^{2+} handling, and secretory granule exocytosis.[27–37]

3.3 SELECTION OF β CELL LINES

High-throughput screening for detection of cAMP in primary cultures of pancreatic β cells is limited by the fact that it is difficult to obtain sufficient numbers of cells for this purpose. However, β cell lines are available, and they provide model systems with which to perform such assays.[38] Three commonly used cell lines are rat INS-1 cells,[39] mouse MIN6 cells,[40] and hamster HIT-T15 cells,[41] all of which are cancer cell lines derived from insulinomas. Additional cell lines are BRIN-D11 cells derived from electrofusion of RINm5f insulinoma cells and primary rat β cells,[42] as well as human insulin–secreting cell lines of β cell origin.[43,44] It is also possible to generate insulin-secreting cells from mouse and human embryonic stem cells,[45–47] although these cell lines have limited insulin secretory capacity. Because insulinoma-derived

insulin-secreting cell lines are transformed and immortalized, they have limitations owing to the fact that they tend to dedifferentiate with increasing passage number.[48,49] Furthermore, some insulin-secreting cell lines do not secrete insulin exclusively, but also secrete islet peptides not normally found in β cells.[50,51] Optimization of an insulin-secreting cell line can be achieved by clonal selection to generate subclones such as INS-1E, INS-1 832/13, and MIN6-K that closely resemble β cells.[52–54] These subclones secrete insulin in response to physiologically relevant concentrations of glucose that span a concentration range of 2.8 to 16.7 mM. Furthermore, these subclones express target GPCRs of interest, in addition to standard genetic markers for β cell glucose sensing such as the Glut2 facilitative glucose transporter and the type IV hexokinase (glucokinase, GK; Figure 3.1). Independent confirmation of a positive *hit* in high-throughput assays using β cell lines is obtainable using human or rodent β cells that are virally transduced with cAMP biosensors so that live-cell imaging can be performed in real time under conditions that allow fluctuations of cAMP concentration to be measured in response to a GPCR agonist.[25]

An additional factor to consider when selecting a β cell line for high-throughput screening is that these cell lines express multiple isoforms of TMACs.[55,56] Ideally, the β cell line that is chosen will replicate the pattern of TMAC expression found in primary β cells. In this regard, rodent β cells express a Ca^{2+}/calmodulin-regulated type 8 adenylyl cyclase (AC-8) that is especially important to the cAMP-dependent stimulation of insulin secretion by GPCR agonists such as GLP-1.[55,57–59] AC-8 acts as a molecular coincidence detector because its activity is stimulated not only by GPCR agonists but also by depolarization-induced Ca^{2+} influx that occurs in response to β cell glucose metabolism.[60–63] Thus, a requirement for glucose metabolism will likely exist when evaluating the efficacy and potency of GPCR agonists to stimulate cAMP production in β cell lines. This requirement can be met using subclones of INS-1 and MIN6 cells that retain their glucose responsiveness.[52–54]

β Cells also express a soluble adenylyl cyclase (sAC) that is activated by the glucose metabolism independently of GPCRs (Figure 3.1).[64,65] The existence of this alternative source of cAMP is understandable because the glucose metabolism in β cells stimulates mitochondrial CO_2 production that upregulates sAC activity. CO_2 is converted to carbonic acid by carbonic anhydrase (CA), and at physiological pH, carbonic acid dissociates to yield bicarbonate ion $\left(HCO_3^-\right)$ that binds to and directly stimulates sAC.[66,67] The CA inhibitor acetazolamide reduces GSIS from isolated islets,[68,69] and a knockout (KO) of sAC gene expression in mice leads to reduced GSIS that is accompanied by reduced glucose tolerance and elevated levels of blood glucose in vivo.[65] Furthermore, the selective sAC inhibitors KH7 and catechol estrogen inhibit GSIS from INS-1E β cells.[64,65] Therefore, the potential role of sAC activity should be taken into consideration when evaluating how investigational compounds stimulate cAMP production in β cell lines.

3.4 BIOSENSORS BASED ON PKA HOLOENZYME DISSOCIATION

The first FRET-based cAMP biosensor was FlCRhR, a reporter developed by the R.Y. Tsien laboratory (Table 3.1).[70] Its design was based on the PKA holoenzyme and

TABLE 3.1
Genetically Encoded Biosensors

	EC_{50} cAMP	References
PKA-Based Biosensors		
FlCRhR (RIα/Cα)	90 nM	Adams et al.[70]
RIIβ EBFP/CαS65T	0.5–0.9 μM	Zaccolo et al.[71]
AKAR3 (ECFP/Venus)	n.a.	Allen and Zhang[72]
AKAR4 (Cerulean/Venus)	n.a.	Depry et al.[73]
Lyn-AKAR4 (Lipid rafts)	n.a.	Depry et al.[73]
AKAR4-Kras (Nonlipid rafts)	n.a.	Depry et al.[73]
PKA-camps (EYFP-RIIβ-ECFP)	1.9 μM	Nikolaev et al.[74]
delta-RIIβ-CFP-CAAX/Cα-YFP	n.r.	Dyachok et al.[75,76]
Epac-Based Biosensors		
Epac1-camps (EYFP-CNBD-ECFP)	2.4 μM	Nikolaev et al.[74]
ICUE1 (ECFP-FL-Epac1-Citrine)	10–50 μM	DiPilato et al.[77]
CFP-FL-Epac1-YFP	50 μM	Ponsioen et al.[78]
CFP-Epac1-ΔDEP-CD-YFP	14 μM	Ponsioen et al.[78]
mCerulean-Epac1-ΔDEP-CD-mCitrine (CEPAC, reduced ion sensitivity)	24 μM	Salonikidis et al.[79]
mTurq.delta-Epac1-[CD, ΔDEP]-cp173-Venus-Venus (expanded dynamic range)	n.r.	Klarenbeek et al.[80]
Epac2-camps (EYFP-CNBD-B-ECFP)	0.9 μM	Nikolaev et al.[74]
Cerulean-FL-Epac2-Venus	n.r.	Herbst et al.[81]
ECFP-FL-Epac2-EYFP	n.r.	Zhang et al.[82]
Citrene-Epac2-camps-CeruleanFP (reduced pH sensitivity)	545 nM	Everett and Cooper[83]
Citrene-Epac2-camps-CeruleanFP-AC8 (fusion to adenylyl cyclase 8)	356 nM	Everett and Cooper[83]

Note: FL, full-length; n.a., not applicable; n.r., not reported. All EC_{50} values are approximate.

incorporates two catalytic subunits (Cα) conjugated to fluorescein, and two regulatory subunits (RIα) conjugated to rhodamine. FlCRhR is not a genetically encoded cAMP biosensor, so it must be introduced into cells as a protein, typically by microinjection. However, understanding the properties of FlCRhR allows an introduction to the genetically encoded cAMP biosensors described below. In FlCRhR, the fluorescein moiety of the Cα subunit (FlC) serves as the donor chromophore for FRET, whereas the rhodamine moiety of the RIα subunit (RhR) serves as the acceptor chromophore. Using 488 nm excitation light, the fluorescein chromophore of FlCRhR is excited while also monitoring the fluorescein and rhodamine emission light intensities at 520 and 580 nm, respectively. In the absence of cAMP, FlCRhR exists as a tetramer ($C\alpha_2RI\alpha_2$) so that FRET will occur between nearby FlC and RhR subunits. However, binding of cAMP to FlCRhR induces its dissociation, and the

resultant decrease of FRET is measurable as an increase of the 520/580 nm emission ratio. Using FlCRhR in combination with the Ca^{2+} indicator fura-2, DeBernardi and Brooker were the first to demonstrate that digital imaging techniques could be used to investigate the Ca^{2+}-dependent control of cAMP biosynthesis and degradation in living cells.[84] Subsequently, Zaccolo et al. designed a genetically encoded cAMP biosensor for use in assays of FRET (Table 3.1).[71] By transfecting cells with cDNAs that directed the expression of fluorescent PKA regulatory (RIIβ) and catalytic (Cα) subunits, Zaccolo et al. achieved the reconstitution of a tetrameric cAMP biosensors in situ.[71] In this biosensor, the RIIβ subunit is a fusion protein incorporating a blue fluorescent protein chromophore (RIIβ-EBFP) serving as the FRET donor, whereas the Cα subunit is fused to a green fluorescent protein chromophore (CαS65T) serving as the FRET acceptor.[71]

With the advent of genetically encoded biosensors, it soon became apparent that compartmentalized cAMP signaling could be monitored using biosensors that incorporate select targeting sequences. For studies of β cells, Dyachok et al. used a bimolecular PKA-based biosensor that does not monitor FRET, but that instead monitors reversible cAMP-dependent translocation of the Cα catalytic subunit from the plasma membrane (PM) to the cytosol (Table 3.1).[75] In this biosensor, the RIIβ subunit is truncated to remove the regulatory subunit dimerization domain and the binding domain for A-kinase anchoring proteins. This truncated ΔRIIβ is fused at its C-terminus to cyan fluorescent protein (CFP), which is itself C-terminally extended to include PM-targeting polybasic and CAAX motifs. The resultant ΔRIIβ-CFP-CAAX fusion protein localizes to the PM where it recruits its molecular partner, a Cα catalytic subunit fused at its C-terminus with yellow fluorescent protein (YFP).

Using total internal reflection microscopy (TIRF), it is possible to image this PKA-based biosensor at the inner surface of the PM, and to also monitor cAMP-induced dissociation of Cα-YFP away from the PM. When combined with the Ca^{2+} indicator fura red, it is possible to detect synchronous oscillations of cAMP and Ca^{2+} that are induced by GLP-1 and that stimulate pulsatile insulin secretion from β cells.[75] Furthermore, it is possible to demonstrate translocation of Cα-YFP from the PM to the nucleus in response to a sustained increase of cAMP, thereby demonstrating a likely role for Cα nuclear translocation in the PKA-dependent control of gene expression.[75] In additional studies using MIN6 cells and mouse β cells, Dyachok et al. also found that glucose, alone, has the capacity to induce oscillations of cAMP occurring immediately beneath the PM.[76] Because this action of glucose is blocked by the TMAC inhibitor 2′,5′-dideoxyadenosine (DDA, a P-site inhibitor), and is reduced by an L-type Ca^{2+} channel blocker (verapamil), it seems possible that β cell glucose metabolism initiates Ca^{2+} influx that activates AC-8 so that oscillations of cAMP are generated.[76]

3.5 BIOSENSORS BASED ON PKA-MEDIATED PHOSPHORYLATION

GPCR signaling pathways are linked to major alterations of PKA activity that potentiate GSIS from β cells.[27,31,37,85–95] Furthermore, PKA holoenzyme complexes containing RIIα regulatory subunits bound to A-kinase anchoring proteins mediate the action of GLP-1 to potentiate GSIS.[96,97] Recently, it was reported that PKA-mediated phosphorylation of the SNARE complex–associated protein Snapin is

one means by which GLP-1 facilitates insulin exocytosis that is Ca^{2+}-dependent.[91] Collectively, these reports are consistent with the finding that GSIS is potentiated by 6-Bn-cAMP-AM, a cAMP analog that activates PKA holoenzyme complexes containing RII but not RI regulatory subunit isoforms (G.G. Holz, unpublished findings). To monitor PKA activity in islets, it is possible to perform immunochemical assays of cAMP-response element-binding protein phosphorylation, or biochemical assays of Kemptide phosphorylation.[25] However, a FRET-based assay using the biosensor AKAR3 allows real-time measurements of PKA activity in single β cells.[25] AKAR3 is a unimolecular A-kinase activity reporter designed by Allen and Zhang (Table 3.1).[72] It consists of an N-terminal CFP chromophore linked to a forkhead-associated phosphoamino acid-binding domain (FHA-D), a consensus PKA substrate motif (LRRATLVD), and a C-terminal YFP chromophore. Phosphorylation of AKAR3 by PKA leads to a conformational change caused by the binding of the FHA-D to the PKA substrate motif containing phosphothreonine. This molecular rearrangement allows increased FRET that is measurable as an increase of the 535/485 nm emission ratio when exciting at 440 nm. Importantly, the AKAR3 coding sequence can be incorporated into adenovirus for its expression in human β cells for live-cell imaging in real time.[25] As discussed in Section 3.13, AKAR3 can also be expressed in cell lines so that it can serve as a sensitive indicator of PKA activity when performing high-throughput assays using a plate-reading spectrofluorimeter equipped with dual emission monochromators.

3.6 BIOSENSORS BASED ON EPAC ACTIVATION

The discovery of the Epac class of cAMP-regulated guanine nucleotide exchange factors in 1998 provided a new explanation for the ability of cAMP to control cellular functions independently of PKA.[98,99] In terms of β cell function, these PKA-independent actions of cAMP were reported to be mediated by Epac2.[100–102] However, recent findings indicate that Epac1 also participates.[103] Epac-mediated actions of cAMP include the stimulation of insulin secretion,[24–28,33,34,90,104–109] the stimulation of Ca^{2+} signaling,[15,25,26,29,104,110–113] the inhibition of ATP-sensitive K^+ channel (K-ATP) function,[15,30,35,110] and the stimulation of glucokinase activity.[17,18] Much of what is known concerning Epac action in the β cells has been revealed through the use of Epac-selective cAMP analogs (ESCAs). These analogs activate Epac proteins but not PKA when they are used at appropriately low concentrations.[107,114–116] Recently, new small molecules that selectively inhibit Epac activation have become available,[117–119] and new strains of Epac KO mice have been reported.[120–122]

Because Epac1 and Epac2 are unimolecular proteins that undergo major conformational changes upon binding of cAMP, they are suitable for the construction of FRET-based biosensors that contain the isolated cyclic nucleotide-binding domain (CNBD) or the full-length (FL) protein (Table 3.1). Nikolaev et al. designed Epac1-camps, a biosensor composed of EYFP-$Epac1_{CNBD}$-ECFP,[74] whereas DiPilato et al. designed ICUE1 composed of ECFP-$Epac1_{FL}$-Citrine.[77] Using a similar strategy, Ponsioen et al. designed CFP-$Epac1_{FL}$-YFP.[78] Other variants exist, including those based on Epac2 such as EYFP-$Epac2_{CNBD}$-ECFP,[74] ECFP-$Epac2_{FL}$-EYFP,[82] or Cerulean-$Epac2_{FL}$-Venus.[81] Second generation cAMP biosensors with improved

properties include those with reduced pH sensitivity,[83] reduced ion sensitivity,[79] and wider dynamic range.[80] Additional features of these Epac-based biosensors are the inclusion of a PM targeting motif for the detection of cAMP at the PM,[73] or fusion of the biosensor with phosphodiesterases (PDEs) to monitor PDE activity.[123]

3.7 MODELING OSCILLATIONS OF cAMP AND Ca^{2+}

Landa et al. were the first to demonstrate that a cAMP biosensor could detect the dynamic processes of cAMP production and degradation in β cells.[56] Furthermore, Landa et al. were the first to demonstrate the dynamic interplay of cAMP and Ca^{2+} in β cells, as studied using Epac1-camps in combination with fura-2.[56,61] These findings of Landa et al. are incorporated into a mathematical model of second messenger interplay developed by Fridlyand et al., in which Ca^{2+} stimulates AC-8 and cyclic nucleotide PDEs to control cAMP synthesis and degradation.[61] The key findings of Landa et al. were that sustained depolarization with KCl leads to sustained Ca^{2+} influx and a sustained increase of cAMP that is explained by Ca^{2+}-dependent activation of AC-8.[56] However, periodic depolarization generates oscillatory Ca^{2+} influx that not only activates AC-8 but also activates PDEs.[56] Thus, under such conditions of periodic depolarization, the levels of cAMP and Ca^{2+} oscillate in a synchronous but antiphasic manner because Ca^{2+}-stimulated PDE activity predominates over Ca^{2+}-stimulated AC-8 activity.[61]

One interesting feature of the model devised by Fridlyand et al. is its prediction that GLP-1 will determine the phase relationships of cAMP and Ca^{2+} oscillations under conditions in which β cells are exposed to glucose. Because high concentrations of glucose induce periodic depolarization of β cells, the model of Fridlyand et al. predicts that glucose, alone, will stimulate synchronous antiphasic oscillations of cAMP and Ca^{2+} due to the fact that PDE activity is elevated.[61] However, such antiphasic oscillations will quickly transition to synchronous in-phase oscillations under conditions in which β cells are simultaneously exposed to GLP-1 and glucose. This GLP-1-induced reversal of the oscillatory pattern is explained by synergistic actions of GLP-1 and glucose to activate AC-8 so that cAMP production will prevail over PDE-catalyzed cAMP degradation. More specifically, synergistic activation of AC-8 is explained by direct binding of G_s proteins and Ca^{2+}/calmodulin to AC-8.[61]

3.8 HIGH-THROUGHPUT DETECTION OF cAMP

Chepurny et al. were the first to report that a cAMP biosensor can be stably expressed in a mammalian cell line so that levels of cAMP can be monitored in assays of FRET using a sensitive plate-reading spectrofluorimeter (FlexStation 3, Molecular Devices, Sunnyvale, CA).[107] This assay was first developed using subclones of INS-1 cells stably expressing Epac1-camps. One subclone designated as C-10 was selected after screening multiple subclones for a ΔFRET in response to cAMP-elevating agents (forskolin, IBMX). It was then established that this approach was applicable to INS-1 cells expressing AKAR3, and also in human embryonic kidney cells (HEK293) expressing Epac1-camps, AKAR3, FL-Epac1, and FL-Epac2.[117,119] A significant advance is our optimization of this assay so that GLP-1R agonists can be screened in a high-throughput mode using HEK293 cells coexpressing the human GLP-1R and

either Epac1-camps or AKAR3. It is expected that this FRET-based assay will be applicable to many cell lines that express GPCRs positively linked to cAMP production. Furthermore, this assay will be useful for screens of small molecules that act intracellularly as cAMP agonists or antagonists.

3.9 INS-1 CELL CULTURE AND TRANSFECTION

INS-1 cells are maintained in a humidified incubator (95% air, 5% CO_2) at 37°C in RPMI 1640 medium containing 10 mM Hepes, 11.1 mM glucose, 10% FBS, 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, and 50 μM 2-mercaptoethanol.[39] INS-1 cells are passaged by trypsinization and subcultured once a week. The INS-1 cell clones stably expressing Epac1-camps are generated by G418 selection after transfection of cells with the Epac1-camps coding sequence in pcDNA3.1.[74] A primary screen is performed to identify an INS-1 cell clone that is highly responsive to cAMP-elevating agents forskolin (2 μM) and IBMX (100 μM). Fluorescence microscopy is used to validate cytosolic expression of Epac1-camps in all cells of an individual clone.

3.10 SPECTROFLUORIMETRY

INS-1 cell clones expressing Epac1-camps are plated at 80% confluence on 96-well clear-bottomed assay plates (Costar 3904). The cells are grown in culture overnight prior to their use. Assays are performed using a FlexStation 3 microplate reader equipped with excitation and emission monochromators, and controlled using SoftMax Pro software (Molecular Devices). On the day of the experiment, the culture medium is replaced with 170 μL/well of a standard extracellular saline (SES) solution containing (in millimoles): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 11.1 glucose, 0.1% DMSO, and 10 Hepes (295 mOsm; pH 7.4). The excitation light is delivered at 435 nm (455 nm cutoff), and the emitted light is detected at 485 nm (CFP) or 535 nm (YFP). The excitation light source is a Xenon flash lamp, and the emission intensities are averages of 12 excitation flashes for each time point. Test solutions containing cAMP-elevating agents are first dissolved in SES solution containing 0.1% DMSO. The test solutions are then aliquoted into individual wells of V-bottomed 96-well plates (Greiner). An automated pipetting procedure is used to transfer 30 μL of each test solution to the assay plate containing the cells to be studied. The test solutions are injected into each well at a pipette height that corresponded to a fluid level of 150 μL, and the rate of injection was 31 μL/s. The CFP/YFP emission ratio is calculated for each well, and values for 8 to 12 wells are averaged for each time point. A running average algorithm embedded within SoftMax Pro is used to obtain time point-to-time point smoothing of the kinetic determinations of FRET over a time course of 400 s. Values of FRET ratios for each time point are quantified as the mean ± SEM. The time course of the change of FRET ratio is plotted after exporting these data to Origin v8.0 (OriginLab). Data are processed in a spreadsheet format for graphical display. Publication quality artwork is generated by exporting these data as Adobe Illustrator files for processing in a desktop publishing format.

3.11 LIVE-CELL IMAGING

Live-cell imaging is used to obtain independent confirmation of findings obtained in plate reader assays. Clonal C-10 INS-1 cells expressing Epac1-camps are plated onto glass coverslips and maintained in culture medium overnight. On the day of the experiment, the coverslips are mounted in an imaging chamber for perifusion with SES containing 0.1% DMSO and various test solutions. Imaging of cells is performed using a Nikon Ti inverted microscope equipped with a N.A. 1.45 TIRF objective (60×), a Photometrics Cascade 512b EMCCD camera (Roper Scientific), and a Chameleon-2 filter set (Chroma Technology Corp.) composed of a D440/20 excitation filter, a 455DCLP dichroic, and D485/40 (CFP) or D535/30 (YFP) emission filters.[25] The excitation light source is a DeltaRam X monochromator (Photon Technology Int.). Ratiometric analysis of the emitted light corresponding to fluorescence originating within a defined region of the cytoplasm was performed using Metafluor v.7.5 software (Molecular Devices).

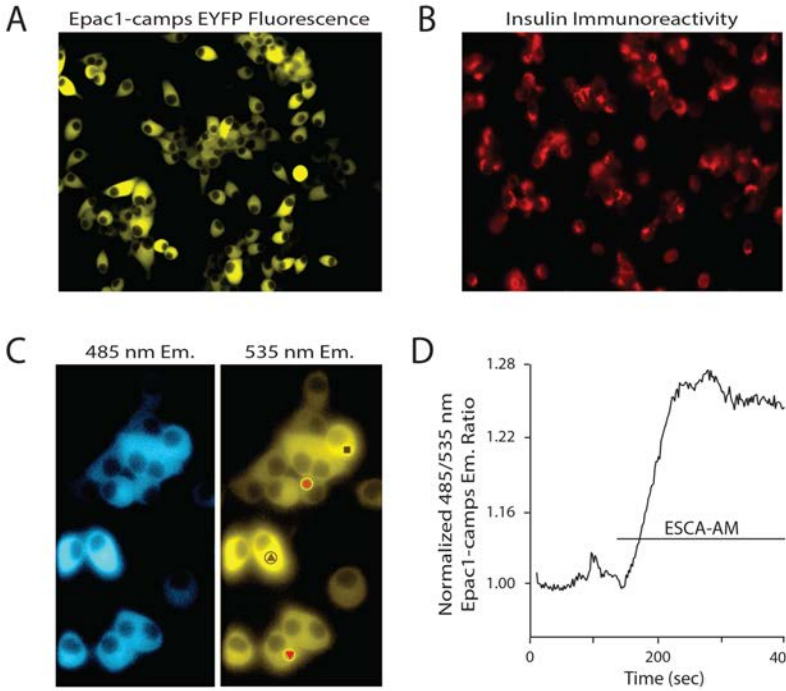

FIGURE 3.2 **(See color insert.)** INS-1 cell clone C-10 stably expresses Epac1-camps, as detected using an EYFP filter set (a). These cells also express insulin immunoreactivity that is detectable by fluorescence immunocytochemistry using an anti-insulin primary antiserum in combination with an Alexa Fluor 594 conjugated secondary antiserum (b). Live-cell imaging of C-10 cells using 440 nm excitation light allows detection of Epac1-camps in which the donor ECFP emission fluorescence is measured at 485 nm, whereas the acceptor EYFP emission fluorescence is measured at 535 nm (c). Activation of Epac1-camps by the Epac-selective cAMP analog 8-pCPT-2′-*O*-Me-cAMP-AM (ESCA-AM, bath application indicated by horizontal line) results in a decrease of FRET that is measured within defined regions of interest (circles over individual cells) as an increase of the 485/535 nm emission ratio (d).

3.12 Epac1-camps VALIDATION

Epac1-camps is stably expressed in monolayers of clone C-10 INS-1 cells that are insulin immunoreactive (Figure 3.2a and b). Activation of Epac1-camps is monitored by live-cell imaging of C-10 cells treated with 8-pCPT-2′-*O*-Me-cAMP-AM applied by bath perifusion (Figure 3.2c and d). This ESCA-AM ester is a prodrug that is highly membrane-permeable and that must undergo intracellular bioactivation so that it can activate Epac proteins.[107,116] Bioactivation of the ESCA-AM is catalyzed by cytosolic esterases that remove the AM moiety so that free 8-pCPT-2′-*O*-Me-cAMP is available.[115] When C-10 cell monolayers are tested in a high-throughput mode using a FlexStation 3 plate reader, Epac1-camps is also activated by 8-pCPT-2′-*O*-Me-cAMP-AM (Figure 3.3a–d). This action of the ESCA-AM is not mimicked by a vehicle solution composed of SES containing 0.1% DMSO (Figure 3.3a) or the negative control AM_3-ester (Figure 3.3d).

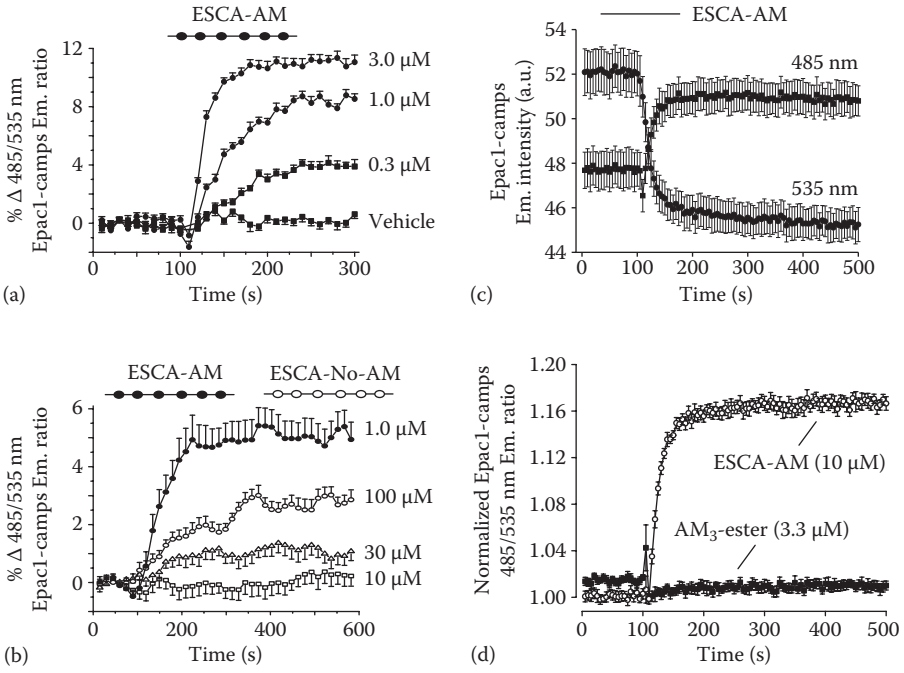

FIGURE 3.3 A FRET-based plate reader assay demonstrates that 8-pCPT-2′-*O*-Me-cAMP-AM (ESCA-AM) dose-dependently activates Epac1-camps in INS-1 cell clone C-10 (a). 8-pCPT-2′-*O*-Me-cAMP not conjugated to the AM-ester has less membrane permeability and a reduced capacity to activate Epac1-camps (b). Epac1-camps activation results in a ΔFRET that is measurable as a decrease of EYFP fluorescence (535 nm) and an increase of ECFP fluorescence (485 nm) (c). The ESCA-AM contains 1 mol of AM-ester per mole of prodrug, and it activates Epac1-camps when tested at a concentration of 10 μM. However, the negative control AM_3-ester contains 3 mol of AM-ester per mole of prodrug and it has no effect when tested at 3.3 μM (d). (Data shown in a and b were originally reported by Chepurny, O.G. et al., *Journal of Biological Chemistry* 284: 10728, 2009.)

8-pCPT-2′-*O*-Me-cAMP that is not conjugated to the AM-ester is at least 100-fold less potent as an activator of Epac1-camps in the FRET assay (Figure 3.3b). This finding is expected because 8-pCPT-2′-*O*-Me-cAMP has limited membrane permeability.[115] However, it can be argued that the action of 8-pCPT-2′-*O*-Me-cAMP-AM reported here is simply an artifact because bioactivation of the analog will generate acetic acid that lowers the cytosolic pH.[115] Because the acceptor chromophore in Epac1-camps is EYFP, it is possible that lowering the pH will quench EYFP fluorescence, thereby yielding an artifactual decrease of FRET unrelated to Epac1-camps activation.[124] This scenario is unlikely because extensive testing of C-10 cells demonstrates that unconjugated AM_3-ester has no effect at Epac1-camps (Figure 3.3d), despite the fact that it is a substrate for cytosolic esterases.[107,116]

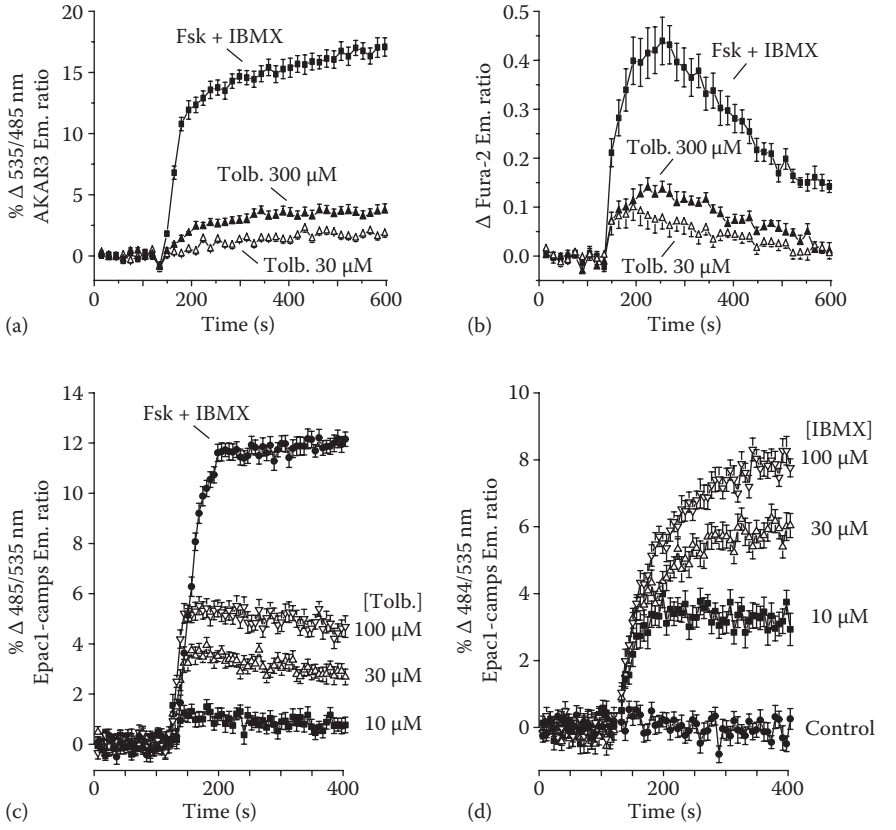

FIGURE 3.4 FRET-based plate reader assays demonstrate that AKAR3 is strongly activated by forskolin (2 μM) and IBMX (100 μM) in INS-1 cells virally transduced with this biosensor. However, tolbutamide produces a weaker effect (a). Fura-2-based plate reader assays performed according to Leech et al.[110] demonstrate that forskolin, IBMX, and tolbutamide also increase the $[Ca^{2+}]_i$ in INS-1 cells (b). When the FRET assay is performed using INS-1 cell clone C-10, Epac1-camps is also activated by forskolin, IBMX, and tolbutamide (c). Note that IBMX alone dose-dependently activates Eapc1-camps in C-10 cells (d).

3.13 AKAR3 VALIDATION

INS-1 cells virally transduced with AKAR3 using the methods of Chepurny et al.[25] provide an alternative means by which to monitor cAMP signaling because AKAR3 detects PKA-mediated phosphorylation. AKAR3 is activated by forskolin and IBMX (Figure 3.4a), and this activation is blocked by 10 μM of the PKA inhibitor H-89 (data not shown). Surprisingly, a high concentration (300 μM) of the sulfonylurea tolbutamide also activates AKAR3 (Figure 3.4a). This action of tolbutamide is accompanied by an increase of $[Ca^{2+}]_i$ in cells loaded with the Ca^{2+} indicator fura-2 (Figure 3.4b). Furthermore, we find that tolbutamide similarly activates Epac1-camps in the INS-1 cell clone C-10 (Figure 3.4c). Such findings seem to indicate that tolbutamide indirectly activates AKAR3 and Epac1-camps by raising the levels of cAMP. This concept is consistent with one prior report in which a high concentration of tolbutamide inhibits PDE activity in lysates derived from islets.[125] For our INS-1 cells, this might also be the case because Epac1-camps is activated by the PDE inhibitor IBMX (Figure 3.4d). Ongoing efforts seek to determine if sulfonylureas exert additional direct actions at cAMP biosensors,[81,82] even though this possibility is currently disputed.[126,127]

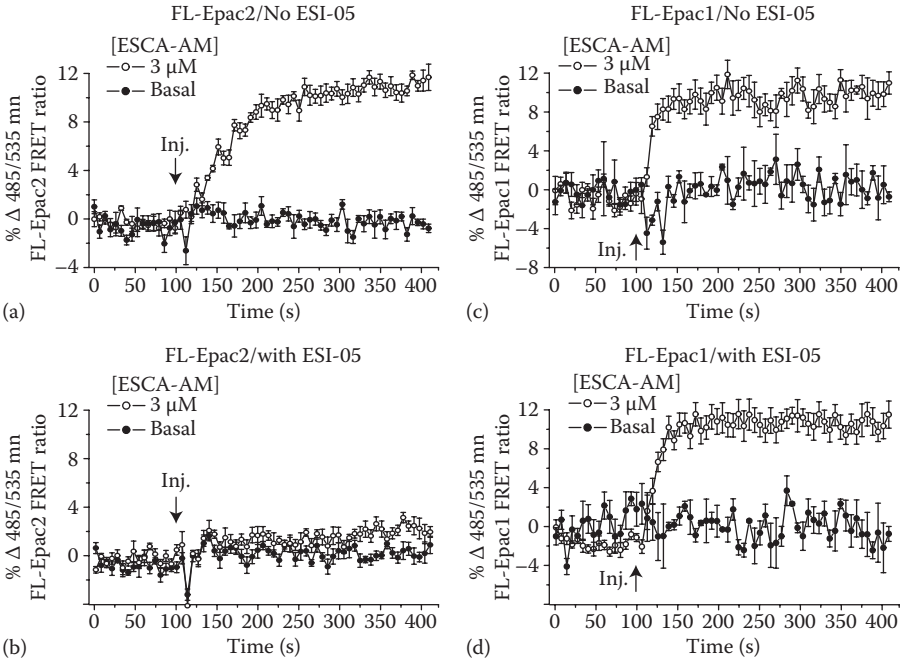

FIGURE 3.5 A full-length Epac2 biosensor (FL-Epac2) stably expressed in HEK293 cell clone C-1 is activated by 3 μM of the ESCA-AM (a), and this activation is blocked by pretreatment of cells with 10 μM of the Epac2-specific inhibitor ESI-05 (b). No such inhibitory action of ESI-05 is measured when a FL-Epac1 biosensor is activated by the ESCA-AM in HEK293 cell clone C-9 (c, d). (All data shown in a to d were originally reported by Tsalkova, T. et al., *Proceedings of the National Academy of Sciences of the United States of America* 109: 18613, 2012.)

3.14 STUDIES WITH HEK293 CELLS

HEK293 cell clones that stably express AKAR3, FL-Epac1, FL-Epac2, and Epac1-camps biosensors are new tools we created to enable high-throughput drug discovery efforts.[117] Evaluation of clones expressing FL-Epac1 (C-9) or FL-Epac2 (C-1) biosensors reveals that these biosensors are activated by forskolin and IBMX, and also by the selective Epac activator 8-pCPT-2′-*O*-Me-cAMP-AM. Remarkably, we find that it is possible to inhibit Epac2 activation using ESI-05, a methylphenylsulfone first discovered by Cheng and coworkers.[117] For example, in the FRET assay, ESI-05 blocks FL-Epac2 but not FL-Epac1 biosensor activation (Figure 3.5a–d). Independent confirmation of this finding is provided by live-cell imaging assays of HEK293 cells stably expressing the FL-Epac2 biosensor (Figure 3.6a and b). Recently, Rehmann[127] used an in vitro Rap1 activation assay to independently confirm the selectivity with which ESI-05 blocks Epac2 activation.

Finally, we report that it is possible to monitor GPCR activation in the plate reader FRET assay using HEK293 cells virally transduced with cAMP biosensors. For example, we use Epac1-camps adenovirus to transduce HEK293 cells that stably

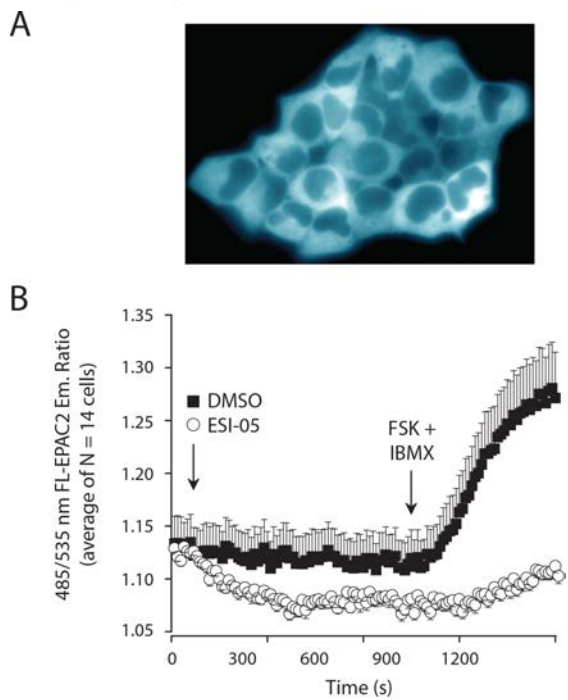

FIGURE 3.6 HEK293 cell clone C-1 expresses the FL-Epac2 biosensor, as imaged in a cluster of cells (a). Live-cell imaging demonstrates that 10 μM ESI-05 blocks activation of FL-Epac2 by 2 μM forskolin and 100 μM IBMX (b). Arrows indicate the times at which test substances were administered by bath perifusion. (All data shown in a and b were originally reported by Tsalkova, T. et al., *Proceedings of the National Academy of Sciences of the United States of America* 109: 18613, 2012.)

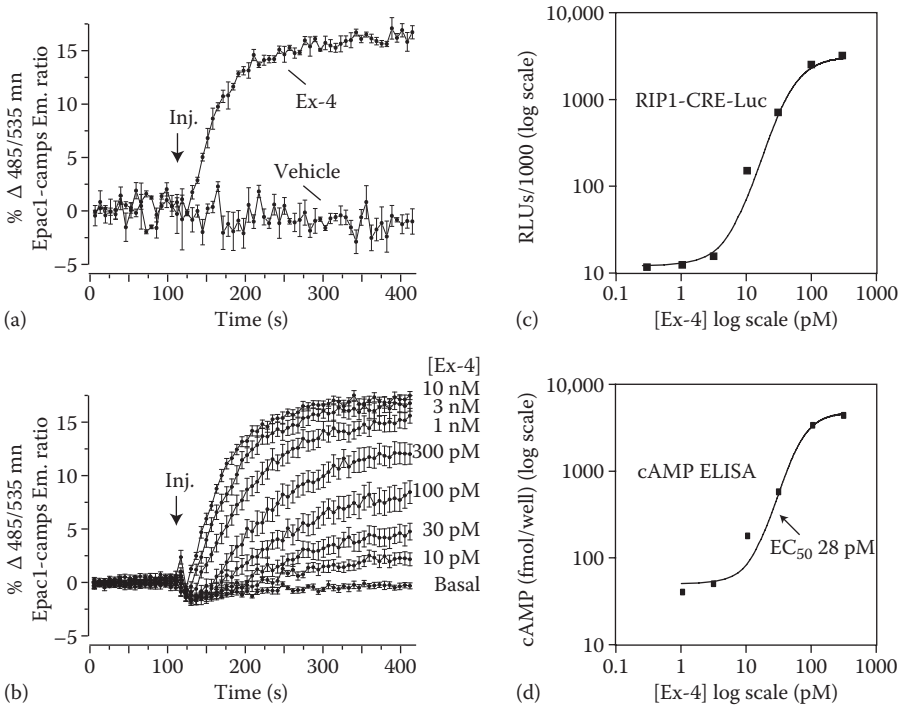

FIGURE 3.7 FRET-based plate reader assays of HEK293 cells stably expressing the GLP-1R and virally transduced with Epac1-camps demonstrate that the GLP-1R agonist exendin-4 (Ex-4, 10 nM) dose-dependently activates Epac1-camps (a, b). Luciferase assays demonstrate that Exendin-4 dose-dependently stimulates CRE-dependent gene expression, as measured in HEK293/GLP-1R cells transfected with RIP1-CRE-Luc (c). ELISA assays demonstrate the exendin-4 dose-dependently stimulates cAMP production in HEK293/GLP-1R cells (d).

express the human GLP-1R.[128] In our FRET assay, the GLP-1R agonist exendin-4 (Ex-4) dose-dependently activates Epac1-camps (Figure 3.7a and b). This action of exendin-4 is most likely cAMP-mediated because exendin-4 also stimulates cAMP-dependent gene transcription that is measurable in HEK293/GLP-1R cells transfected with RIP1-CRE-Luc,[128] a luciferase reporter that incorporates a cAMP response element (Figure 3.7c). Furthermore, an enzyme-linked immunosorbent assay (ELISA) reveals that exendin-4 increases the levels of cAMP in these cells (Figure 3.7d). A cAMP-dependent action of exendin-4 is also evident in HEK293/GLP-1R cells that are virally transduced with AKAR3. In these cells, AKAR3 activation occurs in response to forskolin and IBMX (Figure 3.8a) as well as in exendin-4 (Figure 3.8b). Such actions of forskolin, IBMX, and exendin-4 are nearly eliminated by the PKA inhibitor H-89 (Figure 3.8a, c, and d).

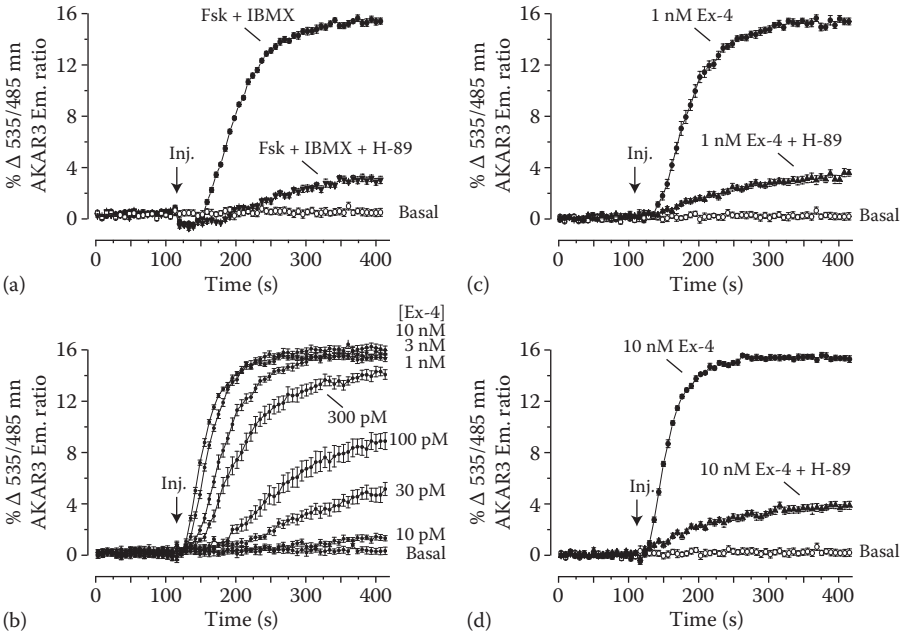

FIGURE 3.8 FRET-based plate reader assays of HEK293 cells stably expressing the GLP-1R and virally transduced with AKAR3 demonstrate that the PKA inhibitor H-89 (10 μM) blocks the actions of forskolin (Fsk, 2 μM) and IBMX (100 μM) to activate AKAR3 (a). The GLP-1R agonist exendin-4 (Ex-4) dose-dependently activates AKAR3 in HEK293/GLP-1R cells (b), and this action of Ex-4 is blocked by 10 μM of the PKA inhibitor H-89 (c, d).

3.15 CONCLUSION

Numerous cAMP biosensors are now available for the assessment of cAMP dynamics in living cells. To date, nearly all published studies using these biosensors have relied on live-cell imaging, as performed using EMCCD cameras interfaced with optics that allow wide-field fluorescence microscopy, confocal microscopy, and TIRF microscopy. Although such approaches allow cAMP to be monitored with fast temporal and high spatial resolution, the use of microscopy is not optimal for assays in which small molecule libraries are screened in a high-throughput mode. Ideally, high-throughput screening should instead be performed in a multiwell format using automated injection of test solutions. Unfortunately, transient transfection with cAMP biosensors does not afford sufficient efficiency to perform low-light level detection of FRET in monolayers of cells. To circumvent this limitation, we generated INS-1 and HEK293 cell clones that stably express AKAR3, Epac1-camps, FL-Epac1, and FL-Epac2 biosensors.[117] Using these clonal cell lines, we demonstrated that it is possible to use a plate-reading spectrofluorimeter in combination with automated injection protocols to perform accurate time-dependent measurements of FRET. Thus,

all cAMP biosensors we tested are highly responsive to cAMP-elevating agents, and we demonstrated that the biosensors Epac1-camps and AKAR3 detect cAMP production in response to GLP-1R activation. Promising areas of future investigation include screens for GLP-1R agonists,[129–131] or screens for molecules that target PKA, Epac1, and Epac2[132–134] Potentially, small molecules might also be identified that target oscillatory circuits in which cAMP, PKA, and Ca^{2+} control insulin secretion.[135] A recent study indicates a prominent role for AC-5 in the control of human islet insulin secretion by glucose,[136] thus this AC isoform might also constitute a relevant target. We conclude that the future remains bright for fluorescence-based assays of cAMP signal transduction as it pertains to small molecule drug discovery.

AUTHOR CONTRIBUTIONS

G.G. Holz wrote the manuscript. C.A. Leech, O.G. Chepurny, and M.W. Roe edited the manuscript. G.G. Holz serves as guarantor of this work.

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REFERENCES

1. Seino, S., T. Shibasaki, and K. Minami. 2011. Dynamics of insulin secretion and the clinical implications for obesity and diabetes. *Journal of Clinical Investigation* 121: 2118.
2. Pessin, J.E., and A.R. Saltiel. 2000. Signaling pathways in insulin action: Molecular targets of insulin resistance. *Journal of Clinical Investigation* 106: 165.
3. Stumvoll, M., B.J. Goldstein, and T.W. van Haeften. 2005. Type 2 diabetes: Principles of pathogenesis and therapy. *Lancet* 365: 1333.
4. Ahrén, B. 2009. Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. *Nature Reviews: Drug Discovery* 8: 369.
5. Furman, B., N. Pyne, P. Flatt, and F. O'Harte. 2004. Targeting beta-cell cyclic 3′5′ adenosine monophosphate for the development of novel drugs for treating type 2 diabetes mellitus. A review. *Journal of Pharmacy and Pharmacology* 56: 1477.
6. Henquin, J.C. 2000. Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49: 1751.
7. Winzell, M.S., and B. Ahrén. 2007. G-protein-coupled receptors and islet function—Implications for treatment of type 2 diabetes. *Pharmacology & Therapeutics* 116: 437.
8. Meier, J.J. 2012. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. *Nature Reviews: Endocrinology* 8: 728.
9. Holz, G.G., and O.G. Chepurny. 2003. Glucagon-like peptide-1 synthetic analogs: New therapeutic agents for use in the treatment of diabetes mellitus. *Current Medicinal Chemistry* 10: 2471.
10. Nadkarni, P., O.G. Chepurny, and G.G. Holz. 2012. Regulation of glucose homeostasis by GLP-1. *Progress in Molecular Biology and Translational Science* 121: 23.

11. Thorens, B. 1992. Expression cloning of the pancreatic β cell receptor for the gluco-incretin hormone glucagon-like peptide-1. *Proceedings of the National Academy of Sciences of the United States of America* 89: 8641.
12. Holz, G.G. 2004. Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic β-cell. *Diabetes* 53: 5.
13. Holz, G.G., G. Kang, M. Harbeck, M.W. Roe, and O.G. Chepurny. 2006. Cell physiology of cAMP sensor Epac. *Journal of Physiology* 577: 5.
14. Leech, C.A., O.G. Chepurny, and G.G. Holz. 2010. Epac2-dependent Rap1 activation and the control of islet insulin secretion by glucagon-like peptide-1. *Vitamins & Hormones* 84: 279.
15. Leech, C.A., I. Dzhura, O.G. Chepurny, G. Kang, F. Schwede, H.G. Genieser et al. 2011. Molecular physiology of glucagon-like peptide-1 insulin secretagogue action in pancreatic β cells. *Progress in Biophysics and Molecular Biology* 107: 236.
16. Holz, G.G., and J.F. Habener. 1992. Signal transduction crosstalk in the endocrine system: Pancreatic β-cells and the glucose competence concept. *Trends in Biochemical Sciences* 17: 388.
17. Ding, S.Y., A. Nkobena, C.A. Kraft, M.L. Markwardt, and M.A. Rizzo. 2011. Glucagon-like peptide 1 stimulates post-translational activation of glucokinase in pancreatic β cells. *Journal of Biological Chemistry* 286: 16768.
18. Park, J.H., S.J. Kim, S.H. Park, D.G. Son, J.H. Bae, H.K. Kim et al. 2012. Glucagon-like peptide-1 enhances glucokinase activity in pancreatic β-cells through the association of Epac2 with Rim2 and Rab3A. *Endocrinology* 153: 574.
19. Holz, G.G. 4th, W.M. Kühtreiber, and J.F. Habener. 1993. Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). *Nature* 361: 362.
20. Dachicourt, N., P. Serradas, D. Bailbé, M. Kergoat, L. Doaré, and B. Portha. 1997. Glucagon-like peptide-1(7–36)-amide confers glucose sensitivity to previously glucose-incompetent β-cells in diabetic rats: In vivo and in vitro studies. *Journal of Endocrinology* 155: 369.
21. Cornu, M., H. Modi, D. Kawamori, R.N. Kulkarni, M. Joffraud, and B. Thorens. 2010. Glucagon-like peptide-1 increases β-cell glucose competence and proliferation by translational induction of insulin-like growth factor-1 receptor expression. *Journal of Biological Chemistry* 285: 10538.
22. Amisten, S., A. Salehi, P. Rorsman, P.M. Jones, and S.J. Persaud. 2013. An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. *Pharmacology & Therapeutics* 139: 359.
23. Sharp, G.W. 1996. Mechanisms of inhibition of insulin release. *American Journal of Physiology—Cell Physiology* 271: C1781.
24. Kelley, G.G., O.G. Chepurny, F. Schwede, H.G. Genieser, C.A. Leech, M.W. Roe et al. 2009. Glucose-dependent potentiation of mouse islet insulin secretion by Epac activator 8-pCPT-2′-*O*-Me-cAMP-AM. *Islets* 1: 260.
25. Chepurny, O.G., G.G. Kelley, I. Dzhura, C.A. Leech, M.W. Roe, E. Dzhura et al. 2010. PKA-dependent potentiation of glucose-stimulated insulin secretion by Epac activator 8-pCPT-2′-*O*-Me-cAMP-AM in human islets of Langerhans. *American Journal of Physiology—Endocrinology and Metabolism* 298: E622.
26. Dzhura, I., O.G. Chepurny, C.A. Leech, M.W. Roe, E. Dzhura, X. Xu et al. 2011. Phospholipase Cε links Epac2 activation to the potentiation of glucose-stimulated insulin secretion from mouse islets of Langerhans. *Islets* 3: 121.
27. Renström, E., L. Eliasson, and P. Rorsman. 1997. Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. *Journal of Physiology* 502: 105.

28. Eliasson, L., X. Ma, E. Renström, S. Barg, P.O. Berggren, J. Galvanovskis et al. 2003. SUR1 regulates PKA-independent cAMP-induced granule priming in mouse pancreatic B-cells. *Journal of General Physiology* 121: 181.
29. Kang, G., O.G. Chepurny, M.J. Rindler, L. Collis, Z. Chepurny, W.H. Li et al. 2005. A cAMP and Ca^{2+} coincidence detector in support of Ca^{2+}-induced Ca^{2+} release in mouse pancreatic β cells. *Journal of Physiology* 566: 173.
30. Kang, G., O.G. Chepurny, B. Malester, M.J. Rindler, H. Rehmann, J.L. Bos et al. 2006. cAMP sensor Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic β cells and rat INS-1 cells. *Journal of Physiology* 573: 595.
31. Hatakeyama, H., T. Kishimoto, T. Nemoto, H. Kasai, and N. Takahashi. 2006. Rapid glucose sensing by protein kinase A for insulin exocytosis in mouse pancreatic islets. *Journal of Physiology* 570: 271.
32. Hatakeyama, H., N. Takahashi, T. Kishimoto, T. Nemoto, and H. Kasai. 2007. Two cAMP-dependent pathways differentially regulate exocytosis of large dense-core and small vesicles in mouse β-cells. *Journal of Physiology* 582: 1087.
33. Shibasaki, T., H. Takahashi, T. Miki, Y. Sunaga, K. Matsumura, M. Yamanaka et al. 2007. Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. *Proceedings of the National Academy of Sciences of the United States of America* 104: 19333.
34. Kwan, E.P., L. Xie, L. Sheu, T. Ohtsuka, and H.Y. Gaisano. 2007. Interaction between Munc13-1 and RIM is critical for glucagon-like peptide-1 mediated rescue of exocytotic defects in Munc13-1 deficient pancreatic β-cells. *Diabetes* 56: 2579.
35. Kang, G., C.A. Leech, O.G. Chepurny, W.A. Coetzee, and G.G. Holz. 2008. Role of the cAMP sensor Epac as a determinant of K_{ATP} channel ATP sensitivity in human pancreatic β-cells and rat INS-1 cells. *Journal of Physiology* 586: 1307.
36. Dzhura, I., O.G. Chepurny, G.G. Kelley, C.A. Leech, M.W. Roe, E. Dzhura et al. 2010. Epac2-dependent mobilization of intracellular Ca^{2+} by glucagon-like peptide-1 receptor agonist exendin-4 is disrupted in β-cells of phospholipase Cε knockout mice. *Journal of Physiology* 588: 4871.
37. Skelin, M., and M. Rupnik. 2011. cAMP increases the sensitivity of exocytosis to Ca^{2+} primarily through protein kinase A in mouse pancreatic beta cells. *Cell Calcium* 49: 89.
38. Skelin, M., M. Rupnik, and A. Cencic. 2010. Pancreatic beta cell lines and their applications in diabetes mellitus research. *Alternatives to Animal Experimentation* 27: 105.
39. Asfari, M., D. Janjic, P. Meda, G. Li, P.A. Halban, and C.B. Wollheim. 1992. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130: 167.
40. Ishihara, H., T. Asano, K. Tsukuda, H. Katagiri, K. Inukai, M. Anai et al. 1993. Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 36: 1139.
41. Santerre, R.F., R.A. Cook, R.M. Crisel, J.D. Sharp, R.J. Schmidt, D.C. Williams et al. 1981. Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cells. *Proceedings of the National Academy of Sciences of the United States of America* 78: 4339.
42. McClenaghan, N.H., and P.R. Flatt. 1999. Engineering cultured insulin-secreting pancreatic B-cell lines. *Journal of Molecular Medicine (Berlin, Germany)* 77: 235.
43. Ravassard, P., Y. Hazhouz, S. Pechberty, E. Bricout-Neveu, M. Armanet, P. Czernichow et al. 2011. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. *Journal of Clinical Investigation* 121: 3589.
44. Scharfmann, R., S. Pechberty, Y. Hazhouz, M. von Bülow, E. Bricout-Neveu, M. Grenier-Godard et al. 2014. Development of a conditionally immortalized human pancreatic β cell line. *Journal of Clinical Investigation* 124: 2087.

45. Li, H., A. Lam, A.M. Xu, K.S. Lam, and S.K. Chung. 2010. High dosage of exendin-4 increased early insulin secretion in differentiated beta cells from mouse embryonic stem cells. *Acta Pharmacologica Sinica* 31: 570.
46. Jiang, W., Y. Shi, D. Zhao, S. Chen, J. Yong, J. Zhang et al. 2007. In vitro derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Research* 17: 333.
47. Bose, B., S.P. Shenoy, S. Konda, and P. Wangikar. 2012. Human embryonic stem cell differentiation into insulin secreting β-cells for diabetes. *Cell Biology International* 36: 1013.
48. Minami, K., H. Yano, T. Miki, K. Nagashima, C.Z. Wang, H. Tanaka et al. 2000. Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines. *American Journal of Physiology—Endocrinology and Metabolism* 279: E773.
49. Cheng, K., V. Delghingaro-Augusto, C.J. Nolan, N. Turner, N. Hallahan, S. Andrikopoulos et al. 2012. High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. *PLoS One* 7: e40868. doi: 10.1371/journal.pone.0040868.
50. Chepurny, O.G., and G.G. Holz. 2002. Over-expression of the glucagon-like peptide-1 receptor on INS-1 cells confers autocrine stimulation of insulin gene promoter activity: A strategy for production of pancreatic β-cell lines for use in transplantation. *Cell and Tissue Research* 307: 191.
51. Nakashima, K., Y. Kanda, Y. Hirokawa, F. Kawasaki, M. Matsuki, and K. Kaku. 2009. MIN6 is not a pure beta cell line but a mixed cell line with other pancreatic endocrine hormones. *Endocrine Journal* 56: 45.
52. Janjic, D., P. Maechler, N. Sekine, C. Bartley, A.S. Annen, and C.B. Wolheim. 1999. Free radical modulation of insulin release in INS-1 cells exposed to alloxan. *Biochemical Pharmacology* 57: 639.
53. Hohmeier, H.E., H. Mulder, G. Chen, R. Henkel-Rieger, M. Prentki, and C.B. Newgard. 2000. Isolation of INS-1-derived cell lines with robust ATP-sensitive K^+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 49: 424.
54. Iwasaki, M., K. Minami, T. Shibasaki, T. Miki, J. Miyazaki, and S. Seino. 2010. Establishment of new clonal pancreatic β-cell lines (MIN6-K) useful for study of incretin/cyclic adenosine monophosphate signaling. *Journal of Diabetes Investigation* 1: 137.
55. Leech, C.A., M.A. Castonguay, and J.F. Habener. 1999. Expression of adenylyl cyclase subtypes in pancreatic β-cells. *Biochemical and Biophysical Research Communications* 254: 703.
56. Landa, L.R. Jr., M. Harbeck, K. Kaihara, O. Chepurny, K. Kitiphongspattana, O. Graf et al. 2005. Interplay of Ca^{2+} and cAMP signaling in the insulin-secreting MIN6 β-cell line. *Journal of Biological Chemistry* 280: 31294.
57. Guenifi, A., G.M. Portela-Gomes, L. Grimelius, S. Efendić, and S.M. Abdel-Halim. 2000. Adenylyl cyclase isoform expression in non-diabetic and diabetic Goto–Kakizaki (GK) rat pancreas. Evidence for distinct overexpression of type-8 adenylyl cyclase in diabetic GK rat islets. *Histochemistry and Cell Biology* 113: 81.
58. Delmeire, D., D. Flamez, S.A. Hinke, J.J. Cali, D. Pipeleers, and F. Schuit. 2003. Type VIII adenylyl cyclase in rat beta cells: Coincidence signal detector/generator for glucose and GLP-1. *Diabetologia* 46: 1383.
59. Roger, B., J. Papin, P. Vacher, M. Raoux, A. Mulot, M. Dubois et al. 2011. Adenylyl cyclase 8 is central to glucagon-like peptide 1 signalling and effects of chronically elevated glucose in rat and human pancreatic beta cells. *Diabetologia* 54: 390.
60. Everett, K.L., and D.M. Cooper. 2013. An improved targeted cAMP sensor to study the regulation of adenylyl cyclase 8 by Ca^{2+} entry through voltage-gated channels. *PLoS One* 8: e75942.

61. Fridlyand, L.E., M.C. Harbeck, M.W. Roe, and L.H. Philipson. 2007. Regulation of cAMP dynamics by Ca^{2+} and G protein-coupled receptors in the pancreatic β-cell: A computational approach. *American Journal of Physiology—Cell Physiology* 293: C1924.
62. Holz, G.G., E. Heart, and C.A. Leech. 2008. Synchronizing Ca^{2+} and cAMP oscillations in pancreatic β-cells: A role for glucose metabolism and GLP-1 receptors? Focus on "regulation of cAMP dynamics by Ca^{2+} and G protein-coupled receptors in the pancreatic β-cell: A computational approach." *American Journal of Physiology—Cell Physiology* 294: C4.
63. Takeda, Y., A. Amano, A. Noma, Y. Nakamura, S. Fujimoto, and N. Inagaki. 2011. Systems analysis of GLP-1 receptor signaling in pancreatic β-cells. *American Journal of Physiology—Cell Physiology* 301: C792.
64. Ramos, L.S., J.H. Zippin, M. Kamenetsky, J. Buck, and L.R. Levin. 2008. Glucose and GLP-1 stimulate cAMP production via distinct adenylyl cyclases in INS-1E insulinoma cells. *Journal of General Physiology* 132: 329.
65. Zippin, J.H., Y. Chen, S.G. Straub, K.C. Hess, A. Diaz, D. Lee et al. 2013. CO_2/HCO_3^- and calcium-regulated soluble adenylyl cyclase as a physiological ATP sensor. *Journal of Biological Chemistry* 288: 33283.
66. Rahman, N., J. Buck, and L.R. Levin. 2013. pH sensing via bicarbonate-regulated *soluble* adenylyl cyclase (sAC). *Frontiers in Physiology* 4: 343.
67. Chang, J.C., and R.P. Oude-Elferink. 2014. Role of the bicarbonate-responsive soluble adenylyl cyclase in pH sensing and metabolic regulation. *Frontiers in Physiology* 5: 42.
68. Parkkila, A.K., A.L. Scarim, S. Parkkila, A. Waheed, J.A. Corbett, and W.S. Sly. 1998. Expression of carbonic anhydrase V in pancreatic beta cells suggests role for mitochondrial carbonic anhydrase in insulin secretion. *Journal of Biological Chemistry* 273: 24620.
69. Sener, A., H. Jijakli, S. Zahedi Asl, P. Courtois, A.P. Yates, S. Meuris et al. 2007. Possible role of carbonic anhydrase in rat pancreatic islets: Enzymatic, secretory, metabolic, ionic, and electrical aspects. *American Journal of Physiology—Endocrinology and Metabolism* 292: E1624.
70. Adams, S.R., A.T. Harootunian, Y.J. Buechler, S.S. Taylor, and R.Y. Tsien. 1991. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* 349: 694.
71. Zaccolo, M., F. De Giorgi, C.Y. Cho, L. Feng, T. Knapp, P.A. Negulescu et al. 2000. A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Nature Cell Biology* 2: 25.
72. Allen, M.D., and J. Zhang. 1998. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochemical and Biophysical Research Communications* 348: 716.
73. Depry, C., M.D. Allen, and J. Zhang. 2011. Visualization of PKA activity in plasma membrane microdomains. *Molecular BioSystems* 7: 52.
74. Nikolaev, V.O., M. Bünemann, L. Hein, A. Hannawacker, and M.J. Lohse. 2004. Novel single chain cAMP sensors for receptor-induced signal propagation. *Journal of Biological Chemistry* 279: 37215.
75. Dyachok, O., Y. Isakov, J. Sågetorp, and A. Tengholm. 2006. Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. *Nature* 439: 349.
76. Dyachok, O., O. Idevall-Hagren, J. Sågetorp, G. Tian, A. Wuttke, C. Arrieumerlou et al. 2008. Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. *Cell Metabolism* 8: 26.
77. DiPilato, L.M., X. Cheng, and J. Zhang. 2004. Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proceedings of the National Academy of Sciences of the United States of America* 101: 16513.

78. Ponsioen, B., J. Zhao, J. Riedl, F. Zwartkruis, G. van der Krogt, M. Zaccolo et al. 2004. Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Reports* 5: 1176.
79. Salonikidis, P.S., M. Niebert, T. Ullrich, G. Bao, A. Zeug, and D.W. Richter. 2011. An ion-insensitive cAMP biosensor for long term quantitative ratiometric fluorescence resonance energy transfer (FRET) measurements under variable physiological conditions. *Journal of Biological Chemistry* 286: 23419.
80. Klarenbeek, J.B., J. Goedhart, M.A. Hink, T.W. Gadella, and K. Jalink. 2011. A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range. *PLoS One* 6: e19170.
81. Herbst, K.J., C. Coltharp, L.M. Amzel, and J. Zhang. 2011. Direct activation of Epac by sulfonylurea is isoform selective. *Chemistry and Biology* 18: 243.
82. Zhang, C.L., M. Katoh, T. Shibasaki, K. Minami, Y. Sunaga, H. Takahashi et al. 2009. The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. *Science* 325: 607.
83. Everett, K.L., and D.M. Cooper. 2013. An improved targeted cAMP sensor to study the regulation of adenylyl cyclase 8 by Ca^{2+} entry through voltage-gated channels. *PLoS One* 8: e75942.
84. DeBernardi, M.A., and G. Brooker. 1996. Single cell Ca^{2+}/cAMP cross-talk monitored by simultaneous Ca^{2+}/cAMP fluorescence ratio imaging. *Proceedings of the National Academy of Sciences of the United States of America* 93: 4577.
85. Harris, T.E., S.J. Persaud, and P.M. Jones. 1997. Pseudosubstrate inhibition of cyclic AMP-dependent protein kinase in intact pancreatic islets: Effects on cyclic AMP-dependent and glucose-dependent insulin secretion. *Biochemical and Biophysical Research Communications* 232: 648.
86. Ding, W.G., and J. Gromada. 1997. Protein kinase A-dependent stimulation of exocytosis in mouse pancreatic β-cells by glucose-dependent insulinotropic polypeptide. *Diabetes* 46: 615.
87. Gromada, J., K. Bokvist, W.G. Ding, J.J. Holst, J.H. Nielsen, and P. Rorsman. 1998. Glucagon-like peptide 1 (7–36) amide stimulates exocytosis in human pancreatic β-cells by both proximal and distal regulatory steps in stimulus-secretion coupling. *Diabetes* 47: 57.
88. McQuaid, T.S., M.C. Saleh, J.W. Joseph, A. Gyulkhandanyan, J.E. Manning-Fox, J.D. MacLellan et al. 2006. cAMP-mediated signaling normalizes glucose-stimulated insulin secretion in uncoupling protein-2 overexpressing β-cells. *Journal of Endocrinology* 190: 669.
89. Goehring, A.S., B.S. Pedroja, S.A. Hinke, L.K. Langeberg, and J.D. Scott. 2007. MyRIP anchors protein kinase A to the exocyst complex. *Journal of Biological Chemistry* 282: 33155.
90. Idevall-Hagren, O., S. Barg, E. Gylfe, and A. Tengholm. 2010. cAMP mediators of pulsatile insulin secretion from glucose-stimulated single β-cells. *Journal of Biological Chemistry* 285: 23007.
91. Song, W.J., M. Seshadri, U. Ashraf, T. Mdluli, P. Mondal, M. Keil et al. 2011. Snapin mediates incretin action and augments glucose-dependent insulin secretion. *Cell Metabolism* 13: 308.
92. Li, N., B. Li, T. Brun, C. Deffert-Delbouille, Z. Mahiout, Y. Daali et al. 2012. NADPH oxidase NOX2 defines a new antagonistic role for reactive oxygen species and cAMP/PKA in the regulation of insulin secretion. *Diabetes* 61: 2842.
93. Nie, J., B.N. Lilley, Y.A. Pan, O. Faruque, X. Liu, W. Zhang et al. 2013. SAD-A potentiates glucose-stimulated insulin secretion as a mediator of glucagon-like peptide 1 response in pancreatic β cells. *Molecular and Cellular Biology* 33: 2527.
94. Kaihara, K.A., L.M. Dickson, D.A. Jacobson, N. Tamarina, M.W. Roe, L.H. Philipson et al. 2013. β-Cell-specific protein kinase A activation enhances the efficiency of glucose control by increasing acute-phase insulin secretion. *Diabetes* 62: 1527.

95. Cognard, E., C.G. Dargaville, D.L. Hay, and P.R. Shepherd. 2013. Identification of a pathway by which glucose regulates β-catenin signalling via the cAMP/protein kinase A pathway in β-cell models. *Biochemical Journal* 449: 803.
96. Lester, L.B., L.K. Langeberg, and J.D. Scott. 1997. Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. *Proceedings of the National Academy of Sciences of the United States of America* 94: 14942.
97. Fraser, I.D., S.J. Tavalin, L.B. Lester, L.K. Langeberg, A.M. Westphal, R.A. Dean et al. 1998. A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. *EMBO Journal* 17: 2261.
98. de Rooij, J., F.J. Zwartkruis, M.H. Verheijen, R.H. Cool, S.M. Nijman, A. Wittinghofer et al. 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396: 474.
99. Kawasaki, H., G.M. Springett, N. Mochizuki, S. Toki, M. Nakaya, M. Matsuda et al. 1998. A family of cAMP-binding proteins that directly activate Rap1. *Science* 282: 2275.
100. Ozaki, N., T. Shibasaki, Y. Kashima, T. Miki, K. Takahashi, H. Ueno et al. 2000. cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nature Cell Biology* 2: 805.
101. Kashima, Y., T. Miki, T. Shibasaki, N. Ozaki, M. Miyazaki, H. Yano et al. 2001. Critical role of cAMP-GEFII—Rim2 complex in incretin-potentiated insulin secretion. *Journal of Biological Chemistry* 276: 46046.
102. Fujimoto, K., T. Shibasaki, N. Yokoi, Y. Kashima, M. Matsumoto, T. Sasaki et al. 2002. Piccolo, a Ca^{2+} sensor in pancreatic β-cells. Involvement of cAMP-GEFII.Rim2. Piccolo complex in cAMP-dependent exocytosis. *Journal of Biological Chemistry* 277: 50497.
103. Kai, A.K., A.K. Lam, Y. Chen, A.C. Tai, X. Zhang, A.K. Lai et al. 2013. Exchange protein activated by cAMP 1 (Epac1)-deficient mice develop β-cell dysfunction and metabolic syndrome. *FASEB Journal* 27: 4122.
104. Kang, G., J.W. Joseph, O.G. Chepurny, M. Monaco, M.B. Wheeler, J.L. Bos et al. 2003. Epac-selective cAMP analog 8-pCPT-2′-O-Me-cAMP as a stimulus for Ca^{2+}-induced Ca^{2+} release and exocytosis in pancreatic β-cells. *Journal of Biological Chemistry* 278: 8279.
105. Ying, Y., L. Li, W. Cao, D. Yan, Q. Zeng, X. Kong et al. 2012. The microtubule associated protein syntabulin is required for glucose-stimulated and cAMP-potentiated insulin secretion. *FEBS Letters* 586: 3674.
106. Idevall-Hagren, O., I. Jakobsson, Y. Xu, and A. Tengholm. 2013. Spatial control of Epac2 activity by cAMP and Ca^{2+}-mediated activation of Ras in pancreatic β cells. *Science Signaling* 6: ra29.1–11, S1–6.
107. Chepurny, O.G., C.A. Leech, G.G. Kelley, I. Dzhura, E. Dzhura, X. Li et al. 2009. Enhanced Rap1 activation and insulin secretagogue properties of an acetoxymethyl ester of an Epac-selective cyclic AMP analog in rat INS-1 cells: Studies with 8-pCPT-2′-O-Me-cAMP-AM. *Journal of Biological Chemistry* 284: 10728.
108. Song, W.J., P. Mondal, Y. Li, S.E. Lee, and M.A. Hussain. 2013. Pancreatic β-cell response to increased metabolic demand and to pharmacologic secretagogues requires EPAC2A. *Diabetes* 62: 2796.
109. Holz, G.G., O.G. Chepurny, and C.A. Leech. 2013. Epac2A makes a new impact in β-cell biology. *Diabetes* 62: 2665.
110. Leech, C.A., I. Dzhura, O.G. Chepurny, F. Schwede, H.G. Genieser, and G.G. Holz. 2010. Facilitation of β-cell K_{ATP} channel sulfonylurea sensitivity by a cAMP analog selective for the cAMP-regulated guanine nucleotide exchange factor Epac. *Islets* 2: 72.
111. Jarrard, R.E., Y. Wang, A.E. Salyer, E.P. Pratt, I.M. Soderling, M.L. Guerra et al. 2013. Potentiation of sulfonylurea action by an EPAC-selective cAMP analog in INS-1 cells: Comparison of tolbutamide and gliclazide and a potential role for EPAC activation of a 2-APB-sensitive Ca^{2+} influx. *Molecular Pharmacology* 83: 191.

112. Kang, G., O.G. Chepurny, and G.G. Holz. 2001. cAMP-regulated guanine nucleotide exchange factor II (Epac2) mediates Ca^{2+}-induced Ca^{2+} release in INS-1 pancreatic β-cells. *Journal of Physiology* 536: 375.
113. Liu, G., S.M. Jacobo, N. Hilliard, and G.H. Hockerman. 2006. Differential modulation of $Ca_v1.2$ and $Ca_v1.3$-mediated glucose-stimulated insulin secretion by cAMP in INS-1 cells: Distinct roles for exchange protein directly activated by cAMP 2 (Epac2) and protein kinase A. *Journal of Pharmacology and Experimental Therapeutics* 318: 152.
114. Enserink, J.M., A.E. Christensen, J. de Rooij, M. van Triest, F. Schwede, H.G. Genieser et al. 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nature Cell Biology* 4: 901.
115. Holz, G.G., O.G. Chepurny, and F. Schwede. 2008. Epac-selective cAMP analogs: New tools with which to evaluate the signal transduction properties of cAMP-regulated guanine nucleotide exchange factors. *Cell Signaling* 20: 10.
116. Vliem, M.J., B. Ponsioen, F. Schwede, W.J. Pannekoek, J. Riedl, M.R. Kooistra et al. 2008. 8-pCPT-2′-O-Me-cAMP-AM: An improved Epac-selective cAMP analogue. *Chembiochem* 9: 2052.
117. Tsalkova, T., F.C. Mei, S. Li, O.G. Chepurny, C.A. Leech, T. Liu et al. 2012. Isoform-specific antagonists of exchange proteins directly activated by cAMP. *Proceedings of the National Academy of Sciences of the United States of America* 109: 18613.
118. Courilleau, D., M. Bisserier, J.C. Jullian, A. Lucas, P. Bouyssou, R. Fischmeister et al. 2012. Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. *Journal of Biological Chemistry* 287: 44192.
119. Chen, H., T. Tsalkova, O.G. Chepurny, F.C. Mei, G.G. Holz, X. Cheng et al. 2013. Identification and characterization of small molecules as potent and specific EPAC2 antagonists. *Journal of Medicinal Chemistry* 56: 952.
120. Yang, Y., X. Shu, D. Liu, Y. Shang, Y. Wu, L. Pei et al. 2012. EPAC null mutation impairs learning and social interactions via aberrant regulation of miR-124 and Zif268 translation. *Neuron* 73: 774.
121. Zhao, K., R. Wen, X. Wang, L. Pei, Y. Yang, Y. Shang et al. 2013. EPAC inhibition of SUR1 receptor increases glutamate release and seizure vulnerability. *Journal of Neuroscience* 33: 8861.
122. Pereira, L., H. Cheng, D.H. Lao, L. Na, R.J. van Oort, J.H. Brown et al. 2013. Epac2 mediates cardiac β1-adrenergic-dependent sarcoplasmic reticulum Ca^{2+} leak and arrhythmia. *Circulation* 127: 913.
123. Herget, S., M.J. Lohse, and V.O. Nikolaev. 2008. Real-time monitoring of phosphodiesterase inhibition in intact cells. *Cell Signaling* 20: 1423.
124. Everett, K.L., and D.M. Cooper. 2012. cAMP measurements with FRET-based sensors in excitable cells. *Biochemical Society Transactions* 40: 179.
125. Goldfine, I.D., R. Perlman, and J. Roth. 1971. Inhibition of cyclic 3′,5′-AMP phosphodiesterase in islet cells and other tissues by tolbutamide. *Nature* 234: 295.
126. Tsalkova, T., A.V. Gribenko, and X. Cheng. 2011. Exchange protein directly activated by cyclic AMP isoform 2 is not a direct target of sulfonylurea drugs. *Assay Drug Development Technologies* 9: 88.
127. Rehmann, H. 2013. Epac-inhibitors: Facts and artefacts. *Scientific Reports* 3: 3032.
128. Chepurny, O.G., and G.G. Holz. 2007. A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts. *Journal of Biomolecular Screening* 12: 740.
129. Holz, G.G., and J.F. Habener. 1998. Black widow spider α-latrotoxin: A presynaptic neurotoxin that shares structural homology with the glucagon-like peptide-1 family of insulin secretagogic hormones. *Comparative Biochemistry and Physiology: Biochemistry and Molecular Biology* 121: 177.

130. Holz, G.G. 2004. New insights concerning the glucose-dependent insulin secretagogue action of glucagon-like peptide-1 in pancreatic β-cells. *Hormone and Metabolic Research* 36: 787.
131. Clardy-James, S., O.G. Chepurny, C.A. Leech, G.G. Holz, and R.P. Doyle. 2013. Synthesis, characterization and pharmacodynamics of vitamin-B_{12}-conjugated glucagon-like peptide-1. *ChemMedChem* 8: 582.
132. Holz, G.G., C.A. Leech, and J.F. Habener. 2000. Insulinotropic toxins as molecular probes for analysis of glucagon-like peptide-1 receptor-mediated signal transduction in pancreatic β-cells. *Biochimie* 82: 915.
133. Chepurny, O.G., C.A. Leech, X. Cheng, and G.G. Holz. 2012. cAMP sensor Epac and gastrointestinal function. In: *Physiology of the Gastrointestinal Tract*, 5th Edition, edited by L. Johnson, F. Ghishan, J.M. Kaunitz, H. Said, and J. Wood, 1849–1861. Oxford: Elsevier.
134. Chen, H., C. Wild, X. Zhou, N. Ye, X. Cheng, and J. Zhou. 2013. Recent advances in the discovery of small molecules targeting exchange proteins directly activated by cAMP (EPAC). *Journal of Medicinal Chemistry* 57: 3651.
135. Ni, Q., A. Ganesan, N.N. Aye-Han, X. Gao, M.D. Allen, A. Levchenko et al. 2011. Signaling diversity of PKA achieved via a Ca^{2+}-cAMP-PKA oscillatory circuit. *Nature Chemical Biology* 7: 34.
136. Hodson, D.J., R.K. Mitchell, L. Marselli, T.J. Pullen, S.G. Brias, F. Semplici et al. 2014. ADCY5 couples glucose to insulin secretion in human islets. *Diabetes* 63: 3009.