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# 3 High-Throughput FRET Assays for Fast Time-Dependent Detection of Cyclic AMP in Pancreatic $\beta$ Cells

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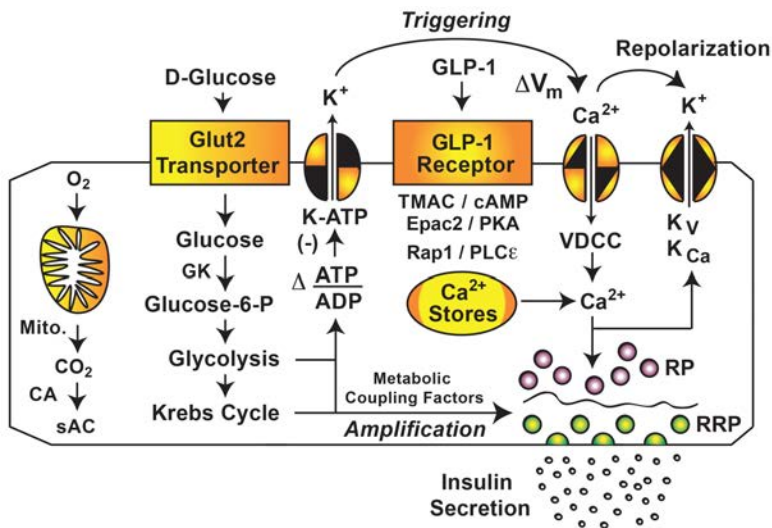
## 3.1 INTRODUCTION

Pancreatic  $\beta$  cells located in the islets of Langerhans secrete insulin in response to the increase of blood glucose concentration that occurs after a meal.<sup>1</sup> 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.<sup>2</sup> 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).<sup>3</sup> 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.<sup>4</sup> 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  $\beta$  cells.<sup>5</sup> We also describe a new fluorescence resonance energy transfer (FRET) assay with which to implement a high-throughput screen of  $\beta$  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 $\beta$ CELLS

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



**FIGURE 3.1** Glucose metabolism in  $\beta$  cells stimulates depolarization ( $\Delta V_m$ ) 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 insulin 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 (PLC $\epsilon$ ). 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.<sup>8</sup> 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  $\beta$  cells.<sup>9</sup> 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.<sup>10</sup>

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  $\beta$  cells (Figure 3.1).<sup>11</sup> 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.<sup>12–15</sup> 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.<sup>12–15</sup> Furthermore, PKA and Epac2 seem to mediate an action of GLP-1R agonists to repair defective stimulus-secretion coupling in the  $\beta$  cells of patients with T2DM.<sup>12–15</sup> Thus, GLP-1R agonists are not simply  $\beta$  cell glucose sensitizers, but they also induce  $\beta$  cell *glucose competence* under conditions of metabolic stress.<sup>16–21</sup> Potentially, the PKA and Epac2 signaling mechanisms in  $\beta$  cells can be targeted to identify new pharmacological agents for use in the treatment of T2DM.

Because  $\beta$  cells express a surprisingly diverse assortment of GPCRs,<sup>22</sup> 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  $\beta$  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).<sup>4,7,22</sup> Because  $\beta$  cells also express GPCRs that inhibit cAMP production,<sup>23</sup> 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,<sup>24–26</sup> or in single-cell assays of  $\beta$  cell ion channel function,  $Ca^{2+}$  handling, and secretory granule exocytosis.<sup>27–37</sup>

### 3.3 SELECTION OF $\beta$ CELL LINES

High-throughput screening for detection of cAMP in primary cultures of pancreatic  $\beta$  cells is limited by the fact that it is difficult to obtain sufficient numbers of cells for this purpose. However,  $\beta$  cell lines are available, and they provide model systems with which to perform such assays.<sup>38</sup> Three commonly used cell lines are rat INS-1 cells,<sup>39</sup> mouse MIN6 cells,<sup>40</sup> and hamster HIT-T15 cells,<sup>41</sup> 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  $\beta$  cells,<sup>42</sup> as well as human insulin-secreting cell lines of  $\beta$  cell origin.<sup>43,44</sup> It is also possible to generate insulin-secreting cells from mouse and human embryonic stem cells,<sup>45–47</sup> 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.<sup>48,49</sup> Furthermore, some insulin-secreting cell lines do not secrete insulin exclusively, but also secrete islet peptides not normally found in  $\beta$  cells.<sup>50,51</sup> 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  $\beta$  cells.<sup>52–54</sup> 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  $\beta$  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  $\beta$  cell lines is obtainable using human or rodent  $\beta$  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.<sup>25</sup>

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

$\beta$  Cells also express a soluble adenylyl cyclase (sAC) that is activated by the glucose metabolism independently of GPCRs (Figure 3.1).<sup>64,65</sup> The existence of this alternative source of cAMP is understandable because the glucose metabolism in  $\beta$  cells stimulates mitochondrial  $\text{CO}_2$  production that upregulates sAC activity.  $\text{CO}_2$  is converted to carbonic acid by carbonic anhydrase (CA), and at physiological pH, carbonic acid dissociates to yield bicarbonate ion ( $\text{HCO}_3^-$ ) that binds to and directly stimulates sAC.<sup>66,67</sup> The CA inhibitor acetazolamide reduces GSIS from isolated islets,<sup>68,69</sup> 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*.<sup>65</sup> Furthermore, the selective sAC inhibitors KH7 and catechol estrogen inhibit GSIS from INS-1E  $\beta$  cells.<sup>64,65</sup> Therefore, the potential role of sAC activity should be taken into consideration when evaluating how investigational compounds stimulate cAMP production in  $\beta$  cell lines.

### 3.4 BIOSENSORS BASED ON PKA HOLOENZYME DISSOCIATION

The first FRET-based cAMP biosensor was FICRhR, a reporter developed by the R.Y. Tsien laboratory (Table 3.1).<sup>70</sup> Its design was based on the PKA holoenzyme and

**TABLE 3.1**  
**Genetically Encoded Biosensors**

	EC <sub>50</sub> cAMP	References
<b>PKA-Based Biosensors</b>		
FICRhr (RI $\alpha$ /C $\alpha$ )	90 nM	Adams et al. <sup>70</sup>
RII $\beta$ EBFP/C $\alpha$ S65T	0.5–0.9 $\mu$ M	Zaccolo et al. <sup>71</sup>
AKAR3 (ECFP/Venus)	n.a.	Allen and Zhang <sup>72</sup>
AKAR4 (Cerulean/Venus)	n.a.	Depry et al. <sup>73</sup>
Lyn-AKAR4 (Lipid rafts)	n.a.	Depry et al. <sup>73</sup>
AKAR4-Kras (Nonlipid rafts)	n.a.	Depry et al. <sup>73</sup>
PKA-camps (EYFP-RII $\beta$ -ECFP)	1.9 $\mu$ M	Nikolaev et al. <sup>74</sup>
delta-RII $\beta$ -CFP-CAAX/C $\alpha$ -YFP	n.r.	Dyachok et al. <sup>75,76</sup>
<b>Epac-Based Biosensors</b>		
Epac1-camps (EYFP-CNBD-ECFP)	2.4 $\mu$ M	Nikolaev et al. <sup>74</sup>
ICUE1 (ECFP-FL-Epac1-Citrine)	10–50 $\mu$ M	DiPilato et al. <sup>77</sup>
CFP-FL-Epac1-YFP	50 $\mu$ M	Ponsioen et al. <sup>78</sup>
CFP-Epac1- $\Delta$ DEP-CD-YFP	14 $\mu$ M	Ponsioen et al. <sup>78</sup>
mCerulean-Epac1- $\Delta$ DEP-CD-mCitrine (CEPAC, reduced ion sensitivity)	24 $\mu$ M	Salonikidis et al. <sup>79</sup>
mTurq.delta-Epac1-[CD, $\Delta$ DEP]-cp173-Venus-Venus (expanded dynamic range)	n.r.	Klarenbeek et al. <sup>80</sup>
Epac2-camps (EYFP-CNBD-B-ECFP)	0.9 $\mu$ M	Nikolaev et al. <sup>74</sup>
Cerulean-FL-Epac2-Venus	n.r.	Herbst et al. <sup>81</sup>
ECFP-FL-Epac2-EYFP	n.r.	Zhang et al. <sup>82</sup>
Citrene-Epac2-camps-CeruleanFP (reduced pH sensitivity)	545 nM	Everett and Cooper <sup>83</sup>
Citrene-Epac2-camps-CeruleanFP-AC8 (fusion to adenylyl cyclase 8)	356 nM	Everett and Cooper <sup>83</sup>

*Note:* FL, full-length; n.a., not applicable; n.r., not reported. All EC<sub>50</sub> values are approximate.

incorporates two catalytic subunits (C $\alpha$ ) conjugated to fluorescein, and two regulatory subunits (RI $\alpha$ ) conjugated to rhodamine. FICRhr 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 FICRhr allows an introduction to the genetically encoded cAMP biosensors described below. In FICRhr, the fluorescein moiety of the C $\alpha$  subunit (FIC) serves as the donor chromophore for FRET, whereas the rhodamine moiety of the RI $\alpha$  subunit (RhR) serves as the acceptor chromophore. Using 488 nm excitation light, the fluorescein chromophore of FICRhr is excited while also monitoring the fluorescein and rhodamine emission light intensities at 520 and 580 nm, respectively. In the absence of cAMP, FICRhr exists as a tetramer (C $\alpha$ <sub>2</sub>RI $\alpha$ <sub>2</sub>) so that FRET will occur between nearby FIC and RhR subunits. However, binding of cAMP to FICRhr induces its dissociation, and the

resultant decrease of FRET is measurable as an increase of the 520/580 nm emission ratio. Using FICRHR in combination with the  $\text{Ca}^{2+}$  indicator fura-2, DeBernardi and Brooker were the first to demonstrate that digital imaging techniques could be used to investigate the  $\text{Ca}^{2+}$ -dependent control of cAMP biosynthesis and degradation in living cells.<sup>84</sup> Subsequently, Zaccolo et al. designed a genetically encoded cAMP biosensor for use in assays of FRET (Table 3.1).<sup>71</sup> By transfecting cells with cDNAs that directed the expression of fluorescent PKA regulatory ( $\text{RII}\beta$ ) and catalytic ( $\text{C}\alpha$ ) subunits, Zaccolo et al. achieved the reconstitution of a tetrameric cAMP biosensors in situ.<sup>71</sup> In this biosensor, the  $\text{RII}\beta$  subunit is a fusion protein incorporating a blue fluorescent protein chromophore ( $\text{RII}\beta$ -EBFP) serving as the FRET donor, whereas the  $\text{C}\alpha$  subunit is fused to a green fluorescent protein chromophore ( $\text{C}\alpha\text{S65T}$ ) serving as the FRET acceptor.<sup>71</sup>

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  $\beta$  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  $\text{C}\alpha$  catalytic subunit from the plasma membrane (PM) to the cytosol (Table 3.1).<sup>75</sup> In this biosensor, the  $\text{RII}\beta$  subunit is truncated to remove the regulatory subunit dimerization domain and the binding domain for A-kinase anchoring proteins. This truncated  $\Delta\text{RII}\beta$  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  $\Delta\text{RII}\beta$ -CFP-CAAX fusion protein localizes to the PM where it recruits its molecular partner, a  $\text{C}\alpha$  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  $\text{C}\alpha$ -YFP away from the PM. When combined with the  $\text{Ca}^{2+}$  indicator fura red, it is possible to detect synchronous oscillations of cAMP and  $\text{Ca}^{2+}$  that are induced by GLP-1 and that stimulate pulsatile insulin secretion from  $\beta$  cells.<sup>75</sup> Furthermore, it is possible to demonstrate translocation of  $\text{C}\alpha$ -YFP from the PM to the nucleus in response to a sustained increase of cAMP, thereby demonstrating a likely role for  $\text{C}\alpha$  nuclear translocation in the PKA-dependent control of gene expression.<sup>75</sup> In additional studies using MIN6 cells and mouse  $\beta$  cells, Dyachok et al. also found that glucose, alone, has the capacity to induce oscillations of cAMP occurring immediately beneath the PM.<sup>76</sup> 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  $\text{Ca}^{2+}$  channel blocker (verapamil), it seems possible that  $\beta$  cell glucose metabolism initiates  $\text{Ca}^{2+}$  influx that activates AC-8 so that oscillations of cAMP are generated.<sup>76</sup>

### 3.5 BIOSENSORS BASED ON PKA-MEDIATED PHOSPHORYLATION

GPCR signaling pathways are linked to major alterations of PKA activity that potentiate GSIS from  $\beta$  cells.<sup>27,31,37,85–95</sup> Furthermore, PKA holoenzyme complexes containing  $\text{RII}\alpha$  regulatory subunits bound to A-kinase anchoring proteins mediate the action of GLP-1 to potentiate GSIS.<sup>96,97</sup> 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  $\text{Ca}^{2+}$ -dependent.<sup>91</sup> 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.<sup>25</sup> However, a FRET-based assay using the biosensor AKAR3 allows real-time measurements of PKA activity in single  $\beta$  cells.<sup>25</sup> AKAR3 is a unimolecular A-kinase activity reporter designed by Allen and Zhang (Table 3.1).<sup>72</sup> It consists of an N-terminal CFP chromophore linked to a forkhead-associated phosphoamino acid-binding domain (FHA-D), a consensus PKA substrate motif (LRRATLVLD), 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  $\beta$  cells for live-cell imaging in real time.<sup>25</sup> 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.<sup>98,99</sup> In terms of  $\beta$  cell function, these PKA-independent actions of cAMP were reported to be mediated by Epac2.<sup>100–102</sup> However, recent findings indicate that Epac1 also participates.<sup>103</sup> Epac-mediated actions of cAMP include the stimulation of insulin secretion,<sup>24–28,33,34,90,104–109</sup> the stimulation of  $\text{Ca}^{2+}$  signaling,<sup>15,25,26,29,104,110–113</sup> the inhibition of ATP-sensitive  $\text{K}^+$  channel (K-ATP) function,<sup>15,30,35,110</sup> and the stimulation of glucokinase activity.<sup>17,18</sup> Much of what is known concerning Epac action in the  $\beta$  cells has been revealed through the use of Epac-selective cAMP analogs (ESCA). These analogs activate Epac proteins but not PKA when they are used at appropriately low concentrations.<sup>107,114–116</sup> Recently, new small molecules that selectively inhibit Epac activation have become available,<sup>117–119</sup> and new strains of Epac KO mice have been reported.<sup>120–122</sup>

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<sub>CNBD</sub>-ECFP,<sup>74</sup> whereas DiPilato et al. designed ICUE1 composed of ECFP-Epac1<sub>FL</sub>-Citrine.<sup>77</sup> Using a similar strategy, Ponsioen et al. designed CFP-Epac1<sub>FL</sub>-YFP.<sup>78</sup> Other variants exist, including those based on Epac2 such as EYFP-Epac2<sub>CNBD</sub>-ECFP,<sup>74</sup> ECFP-Epac2<sub>FL</sub>-EYFP,<sup>82</sup> or Cerulean-Epac2<sub>FL</sub>-Venus.<sup>81</sup> Second generation cAMP biosensors with improved

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

### 3.7 MODELING OSCILLATIONS OF cAMP AND Ca<sup>2+</sup>

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

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<sup>2+</sup> oscillations under conditions in which  $\beta$  cells are exposed to glucose. Because high concentrations of glucose induce periodic depolarization of  $\beta$  cells, the model of Fridlyand et al. predicts that glucose, alone, will stimulate synchronous antiphase oscillations of cAMP and Ca<sup>2+</sup> due to the fact that PDE activity is elevated.<sup>61</sup> However, such antiphase oscillations will quickly transition to synchronous in-phase oscillations under conditions in which  $\beta$  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<sub>s</sub> proteins and Ca<sup>2+</sup>/calmodulin to AC-8.<sup>61</sup>

### 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).<sup>107</sup> 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  $\Delta$ FRET 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.<sup>117,119</sup> 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

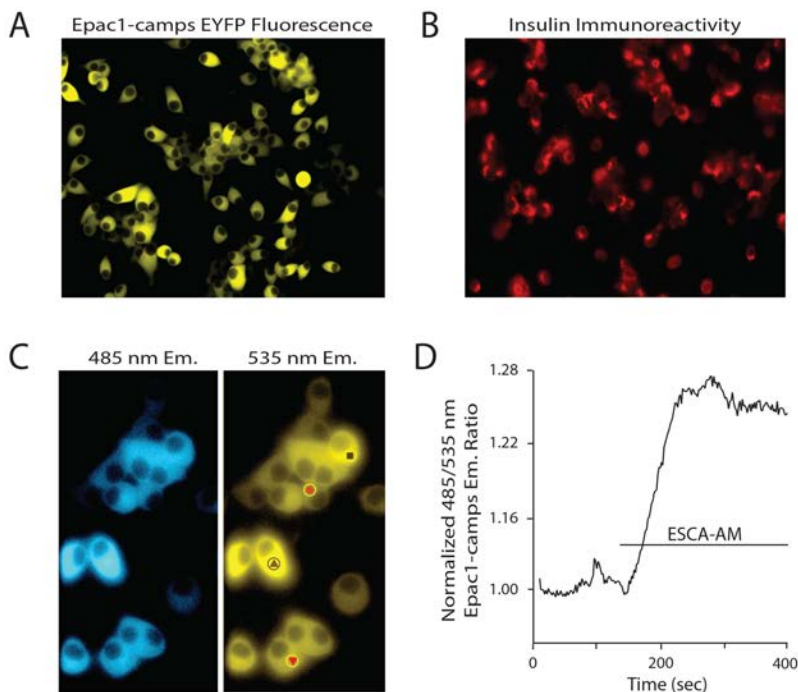
INS-1 cells are maintained in a humidified incubator (95% air, 5% CO<sub>2</sub>) at 37°C in RPMI 1640 medium containing 10 mM Hepes, 11.1 mM glucose, 10% FBS, 100 U mL<sup>-1</sup> penicillin G, 100 µg mL<sup>-1</sup> streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, and 50 µM 2-mercaptoethanol.<sup>39</sup> 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.<sup>74</sup> 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<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 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

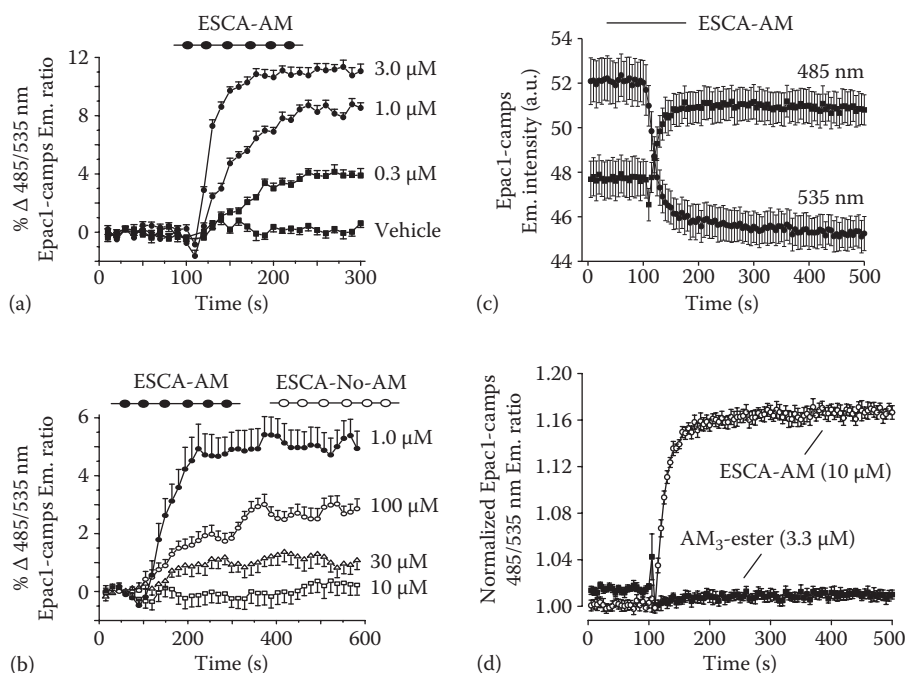
Live-cell imaging is used to obtain independent confirmation of findings obtained in plate reader assays. Clonal C-10 INS-1 cells expressing Epac1-camps are plated onto glass coverslips and maintained in culture medium overnight. On the day of the experiment, the coverslips are mounted in an imaging chamber for perfusion 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 $\times$ ), 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.<sup>25</sup> 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).

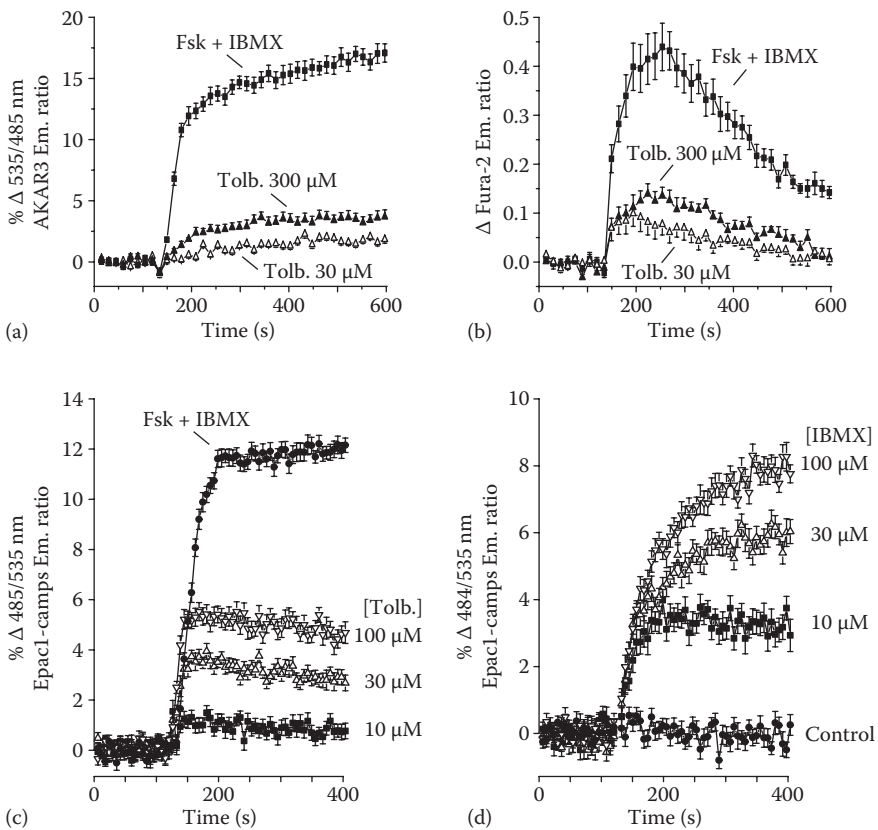
### 3.12 Epacl-camps VALIDATION

Epacl-camps is stably expressed in monolayers of clone C-10 INS-1 cells that are insulin immunoreactive (Figure 3.2a and b). Activation of Epacl-camps is monitored by live-cell imaging of C-10 cells treated with 8-pCPT-2'-*O*-Me-cAMP-AM applied by bath perfusion (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.<sup>107,116</sup> 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.<sup>115</sup> When C-10 cell monolayers are tested in a high-throughput mode using a FlexStation 3 plate reader, Epacl-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<sub>3</sub>-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 Epacl-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 Epacl-camps (b). Epacl-camps activation results in a  $\Delta$ FRET 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 Epacl-camps when tested at a concentration of 10  $\mu$ M. However, the negative control AM<sub>3</sub>-ester contains 3 mol of AM-ester per mole of prodrug and it has no effect when tested at 3.3  $\mu$ M (d). (Data shown in a and b were originally reported by Chepurny, O.G. et al., *Journal of Biological Chemistry* 284: 10728, 2009.)

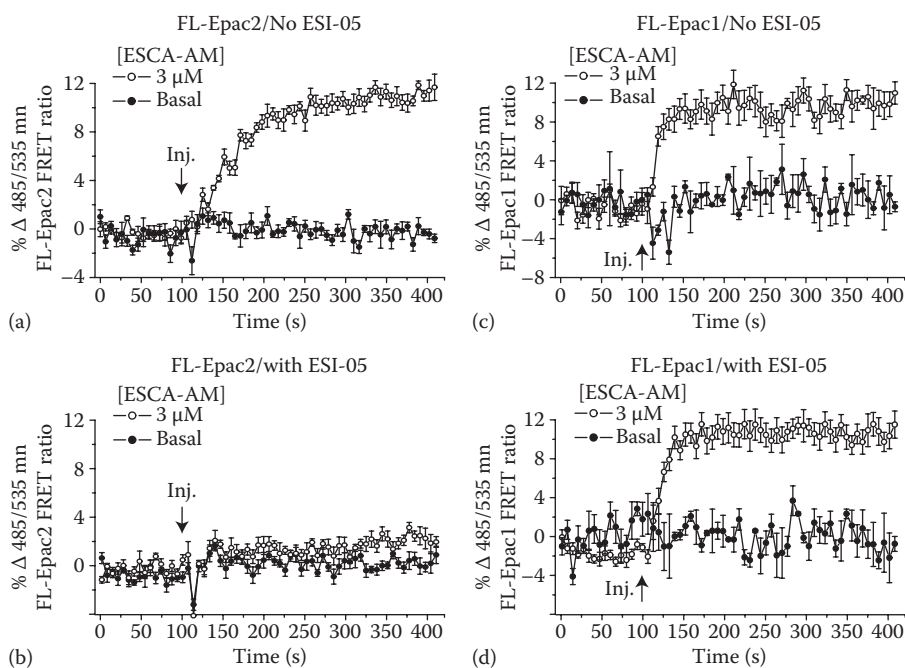
8-pCPT-2'-*O*-Me-cAMP that is not conjugated to the AM-ester is at least 100-fold less potent as an activator of Epac1-camps in the FRET assay (Figure 3.3b). This finding is expected because 8-pCPT-2'-*O*-Me-cAMP has limited membrane permeability.<sup>115</sup> However, it can be argued that the action 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.<sup>115</sup> 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.<sup>124</sup> This scenario is unlikely because extensive testing of C-10 cells demonstrates that unconjugated AM<sub>3</sub>-ester has no effect at Epac1-camps (Figure 3.3d), despite the fact that it is a substrate for cytosolic esterases.<sup>107,116</sup>



**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.<sup>110</sup> 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 Epac1-camps in C-10 cells (d).

### 3.13 AKAR3 VALIDATION

INS-1 cells virally transduced with AKAR3 using the methods of Chepurny et al.<sup>25</sup> 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  $\mu\text{M}$  of the PKA inhibitor H-89 (data not shown). Surprisingly, a high concentration (300  $\mu\text{M}$ ) of the sulfonyleurea tolbutamide also activates AKAR3 (Figure 3.4a). This action of tolbutamide is accompanied by an increase of  $[\text{Ca}^{2+}]_i$  in cells loaded with the  $\text{Ca}^{2+}$  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.<sup>125</sup> 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 sulfonyleureas exert additional direct actions at cAMP biosensors,<sup>81,82</sup> even though this possibility is currently disputed.<sup>126,127</sup>

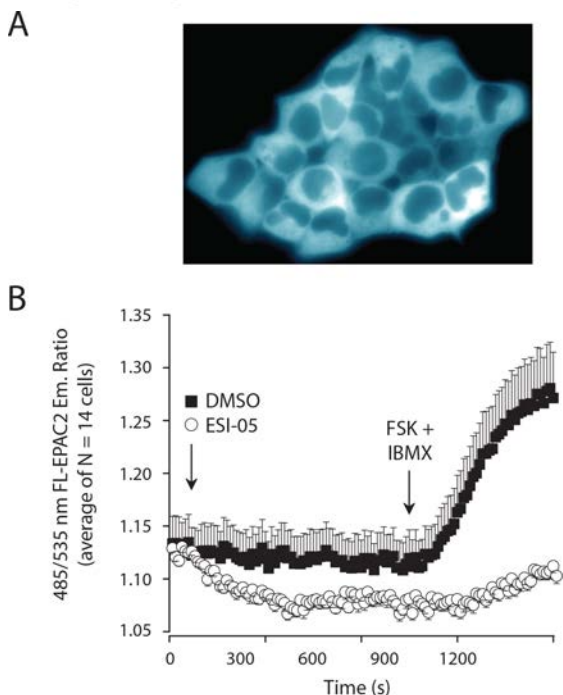


**FIGURE 3.5** A full-length Epac2 biosensor (FL-Epac2) stably expressed in HEK293 cell clone C-1 is activated by 3  $\mu\text{M}$  of the ESCA-AM (a), and this activation is blocked by pretreatment of cells with 10  $\mu\text{M}$  of the Epac2-specific inhibitor ESI-05 (b). No such inhibitory action 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.)

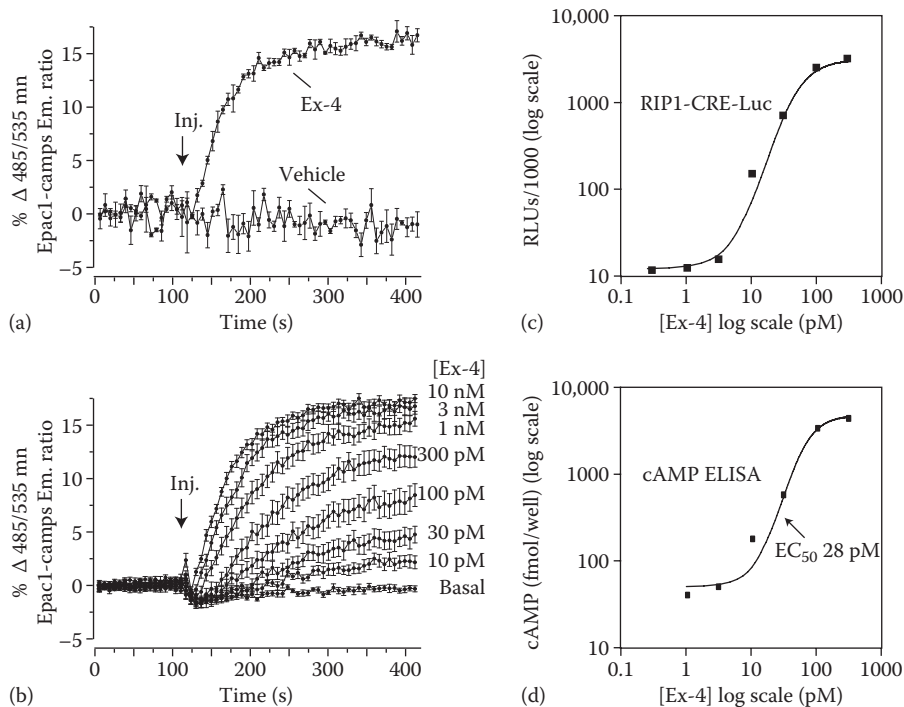
### 3.14 STUDIES WITH HEK293 CELLS

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.<sup>117</sup> 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.<sup>117</sup> 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<sup>127</sup> 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

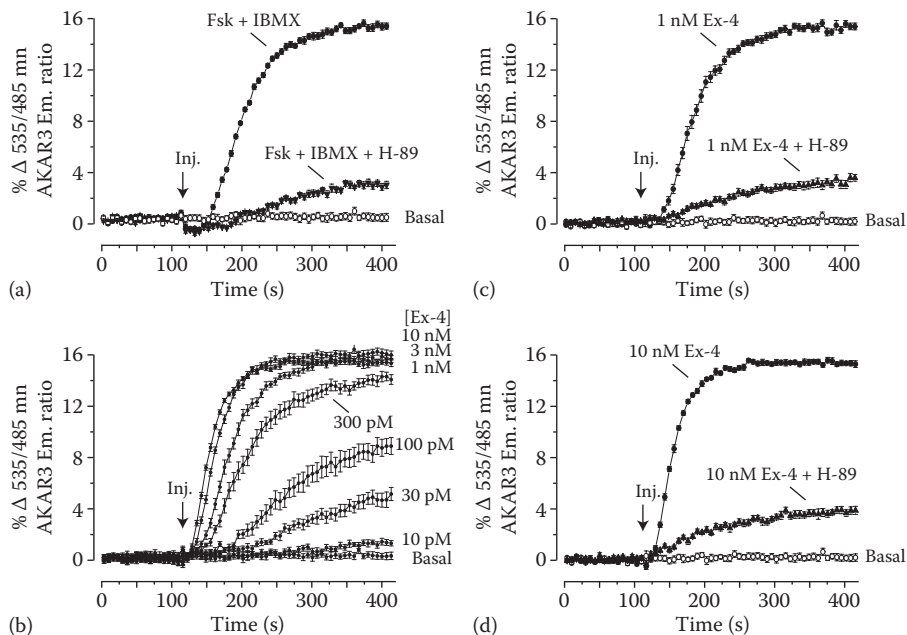


**FIGURE 3.6** HEK293 cell clone C-1 expresses the FL-Epac2 biosensor, as imaged in a cluster of cells (a). Live-cell imaging demonstrates that 10  $\mu$ M ESI-05 blocks activation of FL-Epac2 by 2  $\mu$ M forskolin and 100  $\mu$ M IBMX (b). Arrows indicate the times at which test substances were administered by bath perfusion. (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 Epacl-camps demonstrate that the GLP-1R agonist exendin-4 (Ex-4, 10 nM) dose-dependently activates Epacl-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.<sup>128</sup> In our FRET assay, the GLP-1R agonist exendin-4 (Ex-4) dose-dependently activates Epacl-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,<sup>128</sup> 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  $\mu$ M) blocks the actions of forskolin (Fsk, 2  $\mu$ M) and IBMX (100  $\mu$ M) to activate AKAR3 (a). The GLP-1R agonist extendin-4 (Ex-4) dose-dependently activates AKAR3 in HEK293/GLP-1R cells (b), and this action of Ex-4 is blocked by 10  $\mu$ M 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.<sup>117</sup> 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,<sup>129–131</sup> or screens for molecules that target PKA, Epac1, and Epac2<sup>132–134</sup> Potentially, small molecules might also be identified that target oscillatory circuits in which cAMP, PKA, and Ca<sup>2+</sup> control insulin secretion.<sup>135</sup> A recent study indicates a prominent role for AC-5 in the control of human islet insulin secretion by glucose,<sup>136</sup> 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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