

## Glucose-dependent potentiation of mouse islet insulin secretion by Epac activator 8-pCPT-2'-O-Me-cAMP-AM

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**Abbreviations:** cAMP, adenosine-3',5'-cyclic monophosphate; cAMP-GEF, cAMP-regulated guanine nucleotide exchange factor; CRE, cAMP response element; Epac, exchange protein directly activated by cAMP; ESCA, Epac-selective cAMP analog; GLP-1, glucagon-like peptide-1-(7-36)-amide; GPCR, G protein-coupled receptor; GSIS, glucose-stimulated insulin secretion; K-ATP, ATP-sensitive K<sup>+</sup> channel; PKA, protein kinase A; 8-pCPT-2'-O-Me-cAMP-AM, 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate acetoxymethyl ester

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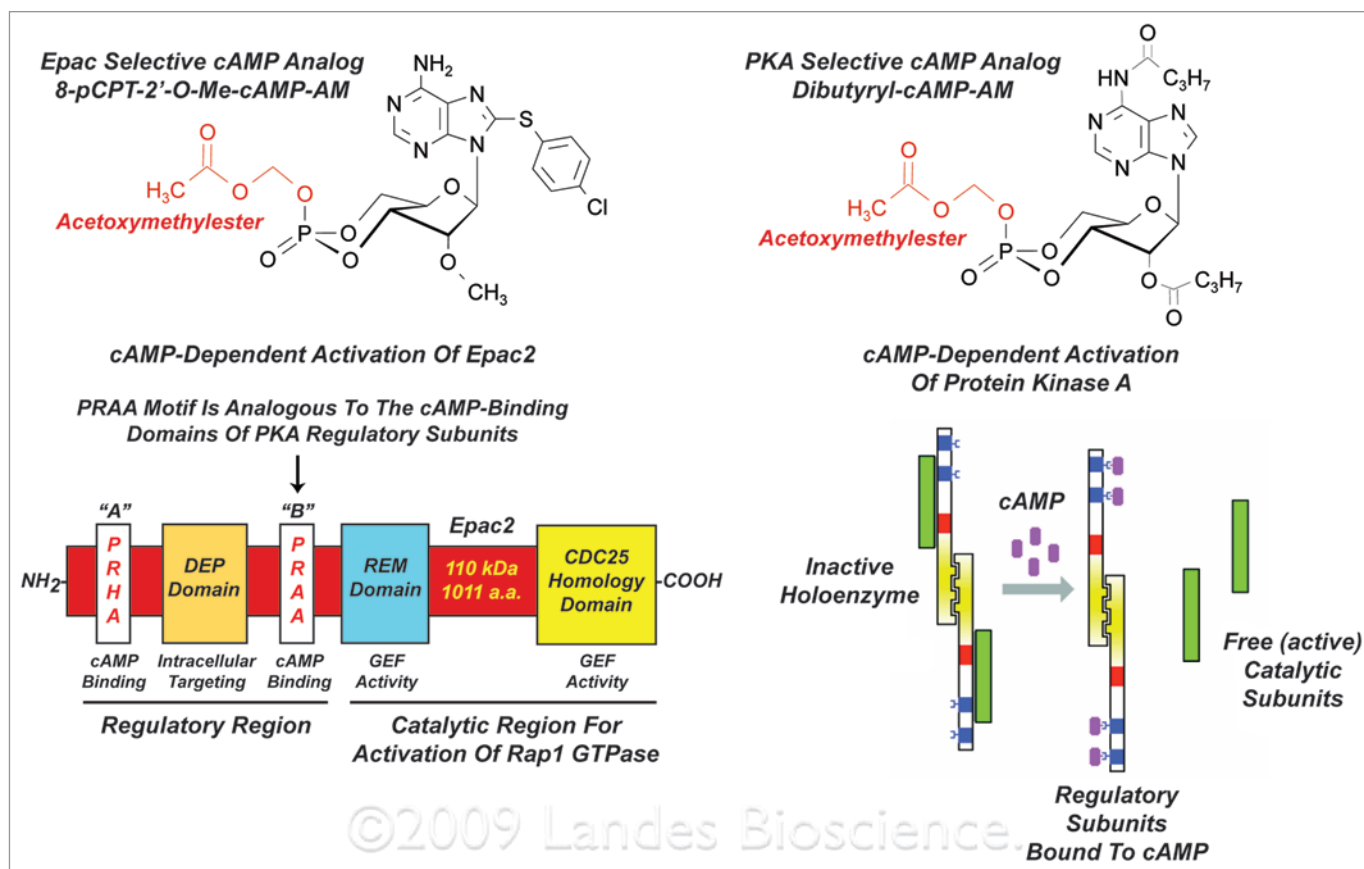
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**E**pac2 is a cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF) that is proposed to mediate stimulatory actions of the second messenger cAMP on mouse islet insulin secretion. Here we have used methods of islet perfusion to demonstrate that the acetoxymethyl ester (AM-ester) of an Epac-selective cAMP analog (ESCA) penetrates into mouse islets and is capable of potentiating both first and second phases of glucose-stimulated insulin secretion (GSIS). When used at low concentrations (1–10  $\mu$ M), 8-pCPT-2'-O-Me-cAMP-AM activates Rap1 GTPase but exhibits little or no ability to activate protein kinase A (PKA), as validated in assays of *in vitro* PKA activity (phosphorylation of Kemptide), Ser<sup>133</sup> CREB phosphorylation status, RIP1-CRE-Luc reporter gene activity, and PKA-dependent AKAR3 biosensor activation. Since quantitative PCR demonstrates Epac2 mRNA to be expressed at levels *ca.* 5.3-fold greater than that of Epac1, available evidence indicates that Epac2 does in fact mediate stimulatory actions of cAMP on mouse islet GSIS.

Glucose-stimulated insulin secretion (GSIS) from the islets of Langerhans is potentiated by glucagon-like peptide-1-(7-36)-amide (GLP-1), an incretin hormone released from entero-endocrine L-cells of the distal intestine in response to the ingestion of a meal.<sup>1-4</sup> GLP-1 binds to a Class II G protein-coupled receptor (GPCR) expressed on pancreatic  $\beta$ -cells,<sup>5</sup> and it stimulates cAMP production,<sup>6</sup> thereby

potentiating stimulatory effects of glucose metabolism on islet insulin secretion.<sup>7-9</sup> In view of the fact that GLP-1 exerts a blood glucose-lowering effect in type 2 diabetic subjects,<sup>1-4</sup> it is of interest to identify the cAMP-regulated signal transduction pathways that are activated as a consequence of the binding of GLP-1 to its GPCR.<sup>10-12</sup> In a recently published paper we reported a new technical advance that may further this goal and which involves the use of an acetoxymethyl ester of an Epac-selective cAMP analog (ESCA) designated as 8-pCPT-2'-O-Me-cAMP-AM.<sup>13</sup>

Since GLP-1 receptor (GLP-1-R) activation raises levels of cAMP in  $\beta$ -cells, it was originally assumed that the insulin secretagogue action of this hormone resulted from its ability to activate protein kinase A (PKA). Unexpectedly, it was reported that there exists in  $\beta$ -cells a novel cAMP signaling mechanism that does not involve PKA but which instead involves the cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF) designated as Epac2.<sup>14-26</sup> Through the use of a cAMP analog that is a selective activator of Epac,<sup>27</sup> it was reported that Epac activation stimulates  $\beta$ -cell mitochondrial ATP production,<sup>28</sup> inhibits ATP-sensitive K<sup>+</sup> channel (K-ATP) activity,<sup>23,25</sup> promotes Ca<sup>2+</sup> influx and the mobilization of intracellular Ca<sup>2+</sup>,<sup>17,18,22,24</sup> while also “priming” secretory granules, thereby rendering them competent to undergo exocytosis.<sup>29-31</sup> Subsequently, Seino and co-workers reported that Epac2 couples  $\beta$ -cell cAMP production to the activation of Rap1, a GTPase Seino found to play an essential



**Figure 1.** Acetoxymethyl esters of Epac and PKA selective cAMP analogs. 8-pCPT-2'-O-Me-cAMP-AM (top left) is hydrolyzed to 8-pCPT-2'-O-Me-cAMP and binds primarily to the high-affinity cAMP binding domain of Epac2 (bottom left). This binding activates the CDC25 homology domain that is responsible for the catalysis of guanyl nucleotide exchange on Rap1. Dibutryryl cAMP-AM (top right; also known as N<sup>6</sup>-2'-DB-cAMP) is hydrolyzed to dibutryryl-cAMP and then to monobutryryl-cAMP which binds to the regulatory subunits of the PKA holoenzyme (bottom right). This binding induces dissociation of the holoenzyme, thereby releasing catalytic subunits with serine/threonine protein kinase activity.

role in the cAMP-dependent stimulation of insulin secretion.<sup>26</sup> How Rap1 might stimulate insulin secretion is not yet fully understood, but we have proposed that it is explained by the ability of Epac2 to act through Rap1 to stimulate the activity of a novel phospholipase C-epsilon that is expressed in  $\beta$ -cells (Kelley GG, unpublished observations) and that couples cAMP production to phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) hydrolysis, protein kinase C (PKC) activation, and the generation of IP<sub>3</sub>, a Ca<sup>2+</sup>-mobilizing second messenger.<sup>32-34</sup> It is important to note, however, that it has yet to be firmly established that Epac2 is of major importance to GLP-1 receptor signal transduction and  $\beta$ -cell stimulus-secretion coupling. In particular, it remains to be determined what the relative contributions of Epac2 and PKA are to the cAMP-dependent potentiation of GSIS. It is also not clear

whether Epac2 and PKA act synergistically to stimulate islet insulin secretion, nor is it established whether the prosecretagogue action of Epac2 is contingent on PKA activation, or vice versa.

To more fully understand what role Epac2 might play in  $\beta$ -cell stimulus-secretion coupling, our laboratory was one of the first to assess potential insulin secretagogue properties of 8-pCPT-2'-O-Me-cAMP. Although this ESCA is *not* an acetoxymethyl ester, it is a selective activator of the two isoforms of Epac known to exist (Epac1, Epac2).<sup>27,35,36</sup> The selectivity with which 8-pCPT-2'-O-Me-cAMP activates Epac proteins is a consequence of the incorporation of a 2'-O-methyl moiety on the ribose ring of cAMP, a modification that drastically reduces the affinity of this compound for PKA relative to its affinity for Epac.<sup>27,37</sup> In studies of human  $\beta$ -cells we demonstrated that

extracellularly-applied 8-pCPT-2'-O-Me-cAMP acted independently of PKA to mobilize an intracellular source of Ca<sup>2+</sup>, thereby raising levels of cytosolic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>c</sub>).<sup>18</sup> Using electrophysiological methods of carbon fiber amperometry, we also found that 8-pCPT-2'-O-Me-cAMP stimulated a brief burst of exocytosis in human  $\beta$ -cells, an action coincident with a transient increase of [Ca<sup>2+</sup>]<sub>c</sub>.<sup>18</sup> Such findings seemed to be in accord with the prior study of Renstrom and co-workers in which cAMP was demonstrated to act in both a PKA-dependent and PKA-independent manner to stimulate exocytosis, as measured by an increase of membrane capacitance in mouse  $\beta$ -cells.<sup>38</sup> Indeed, we concluded that an Epac2-mediated action of cAMP to stimulate exocytosis could explain one previous report in which cAMP-elevating agents were found to act independently of

PKA to exert a time-dependent potentiation of GSIS.<sup>39</sup> Thus, we were surprised to find that 8-pCPT-2'-O-Me-cAMP exhibited no detectable ability to potentiate GSIS in mouse islets, as measured through the use of an insulin-specific radioimmunoassay.<sup>13</sup>

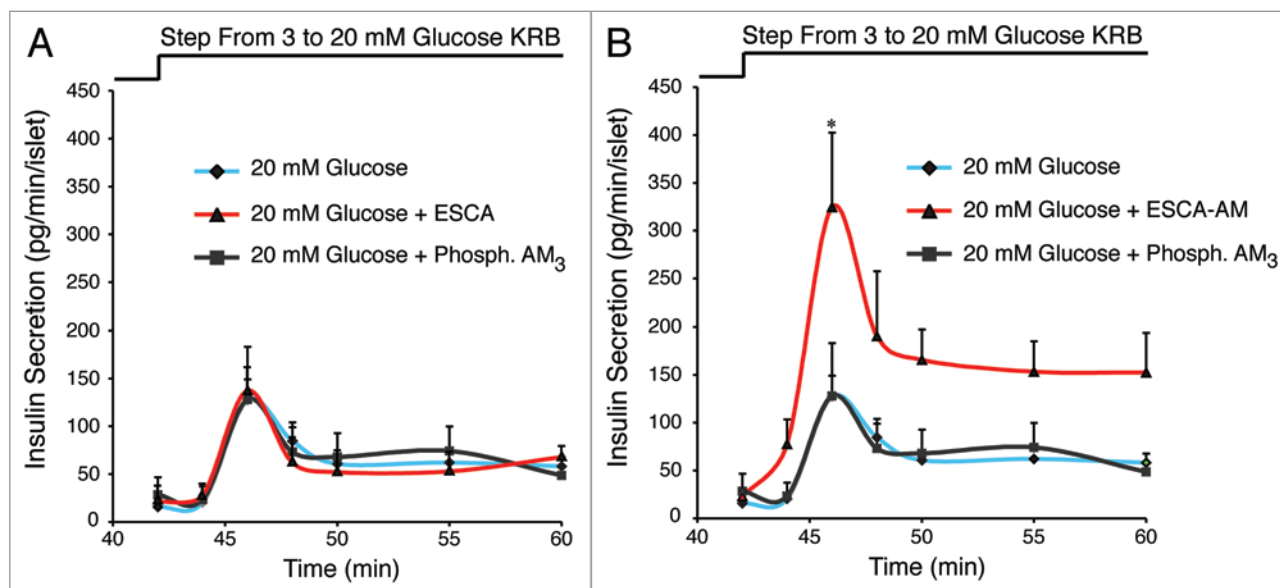
In retrospect it is notable that earlier published reports hinted at a potential complication associated with the use of 8-pCPT-2'-O-Me-cAMP. It was reported that this ESCA failed to potentiate GSIS from mouse islets, even when tested at a concentration of 250  $\mu\text{M}$ .<sup>40</sup> However, a stimulation of insulin secretion was observed in response to 50  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP when rat islets were permeabilized with alpha-toxin,<sup>41</sup> a procedure that allows access of 8-pCPT-2'-O-Me-cAMP to the intracellular compartment of  $\beta$ -cells. Furthermore, direct application of 8-pCPT-2'-O-Me-cAMP to the cytosol of  $\beta$ -cells by use of the patch clamp technique resulted in a potentiation of depolarization-induced exocytosis, as determined by the measurement of membrane capacitance.<sup>29</sup> Thus, it may be concluded that there exists a permeability barrier, one that limits

access of 8-pCPT-2'-O-Me-cAMP to the cytosol of  $\beta$ -cells, but that is not present in cell types in which extracellularly-applied 8-pCPT-2'-O-Me-cAMP exerts its effects.<sup>34,37,42,43</sup> Evidently, this permeability barrier allows small amounts of extracellularly-applied 8-pCPT-2'-O-Me-cAMP to enter  $\beta$ -cells, thereby explaining why a brief burst of exocytosis can be measured when using electrophysiological techniques that afford high temporal resolution.<sup>18</sup>

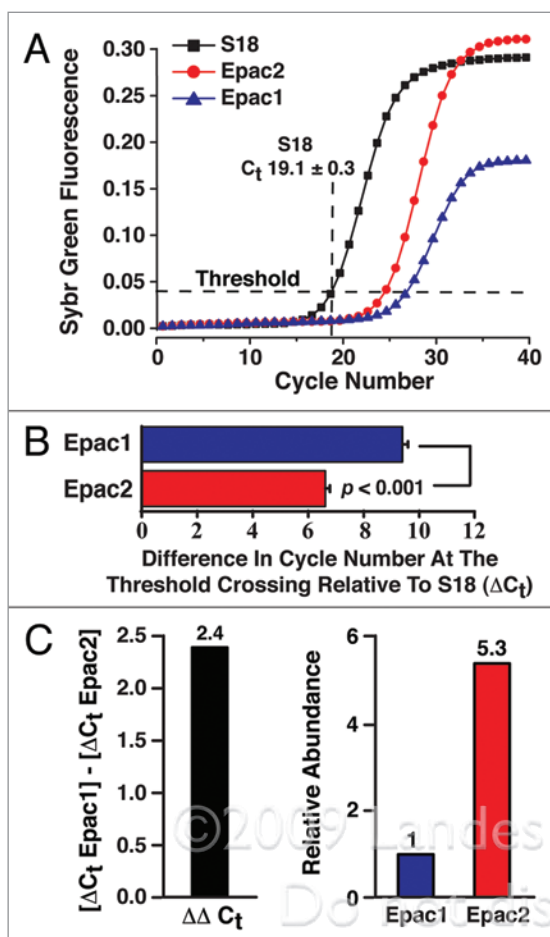
It should be noted that there exists an alternative explanation concerning why 8-pCPT-2'-O-Me-cAMP has little or no efficacy in conventional radioimmunoassays of islet insulin secretion. Using two-photon extracellular polar-tracer (TEP) imaging techniques, Kasai and co-workers reported that 8-pCPT-2'-O-Me-cAMP stimulated the exocytosis of small diameter secretory vesicles from mouse  $\beta$ -cells, whereas it failed to stimulate exocytosis of large diameter dense core vesicles.<sup>44</sup> In contrast, the naturally occurring second messenger cAMP stimulated the exocytosis of large diameter dense core vesicles, and this effect was blocked by Rp-cAMPS, an inhibitor of PKA activation. Since large

dense core vesicles, not small vesicles, are the source of secreted insulin, it was concluded that it is PKA that is the principal signal transducer supporting stimulatory effects of cAMP on insulin secretion.<sup>44</sup> This conclusion is supported by additional published findings.<sup>45-50</sup>

With this summarized information in mind we decided to evaluate potential insulin secretagogue properties of a newly developed cAMP analog that gains access to the cytosol of  $\beta$ -cells in an unimpeded manner. This cAMP analog is 8-pCPT-2'-O-Me-cAMP-AM (Fig. 1). It is an acetoxymethyl ester (AM-ester) of 8-pCPT-2'-O-Me-cAMP and it is highly lipophilic, gaining access to the cytosol in an inactive "pro-drug" form where it is activated by cytosolic esterases that hydrolytically remove the AM-ester moiety.<sup>51</sup> In side-by-side comparisons with 8-pCPT-2'-O-Me-cAMP, we demonstrated that 8-pCPT-2'-O-Me-cAMP-AM was *ca.* 500-fold more potent as an activator of the cAMP biosensor Epac1-camps expressed in rat INS-1 cells, as expected if this ESCA gains ready access to the cytosol.<sup>13</sup> Furthermore, as expected for a selective Epac activator, 8-pCPT-2'-O-Me-cAMP-AM stimulated



**Figure 2.** Differential insulin secretagogue properties of Epac-selective cAMP analogs in adult male C57BL/6J mouse islets. (A) The non-AM-ester of 8-pCPT-2'-O-Me-cAMP (ESCA; 10  $\mu\text{M}$ ) failed to potentiate GSIS induced by a step-wise increase of glucose concentration from 3 to 20 mM. (B) GSIS induced by 20 mM glucose was potentiated by the AM-ester of 8-pCPT-2'-O-Me-cAMP (ESCA-AM; 10  $\mu\text{M}$ ) whereas phosphate-AM<sub>3</sub> (3.3  $\mu\text{M}$ ) was without effect (phosphate-AM<sub>3</sub> liberates 3 mole equivalents of acetic acid and formaldehyde per mole of phosphate when it is hydrolyzed by intracellular esterases). Note that 8-pCPT-2'-O-Me-cAMP-AM was included for 10 min in the KRB perfusate containing 3 mM glucose prior to switching to a perfusate containing 20 mM glucose. Since the initial rate of insulin secretion measured in the presence of 8-pCPT-2'-O-Me-cAMP-AM did not differ from that measured in its absence, it is concluded that 8-pCPT-2'-O-Me-cAMP-AM potentiated GSIS at a high but not a low concentration of glucose. For the methods of analyses, see the Supplementary Data of Chepurny et al. 2009.<sup>13</sup>



**Figure 3.** QPCR for Epac1 and Epac2 mRNA expression in adult male C57BL/6J mouse islets. (A) QPCR fluorescence growth curves from 100 ng of mouse islet RNA using Quiagen QuantiTect Sybr Green RT-PCR. Ribosomal S18 mRNA was used as the reference target for quantification of Epac1 and Epac2 mRNA. The average threshold crossing value ( $C_t$ ) for S18 mRNA ( $19.1 \pm 0.3$ ,  $n = 8$ ; mean  $\pm$  s.e.m.) is indicated. (B) Comparison of the delta- $C_t$  values ( $\Delta C_t$ ) for Epac1 and Epac2. The  $\Delta C_t$  value for each Epac isoform was calculated as the difference in threshold cycle number relative to S18. (C) The  $\Delta\Delta C_t$  value for Epac1 relative to Epac2 was computed by subtraction of the  $\Delta C_t$  values for each isoform (left) and the relative abundance of Epac1 and Epac2 mRNA was then calculated to be 1:5.3 (right).

insulin secretion from INS-1 cells, an effect still measurable after treatment of these cells with 3  $\mu$ M of the PKA inhibitor H-89.<sup>13</sup> This secretagogue action of 8-pCPT-2'-O-Me-cAMP-AM was not reproduced by the non-AM ester of 8-pCPT-2'-O-Me-cAMP, thereby demonstrating that a permeability barrier preventing entry of the ESCA most likely exists in this cell type. Similarly, we found that 8-pCPT-2'-O-Me-cAMP failed to stimulate insulin secretion from mouse islets, whereas 8-pCPT-2'-O-Me-cAMP-AM was effective.<sup>13</sup>

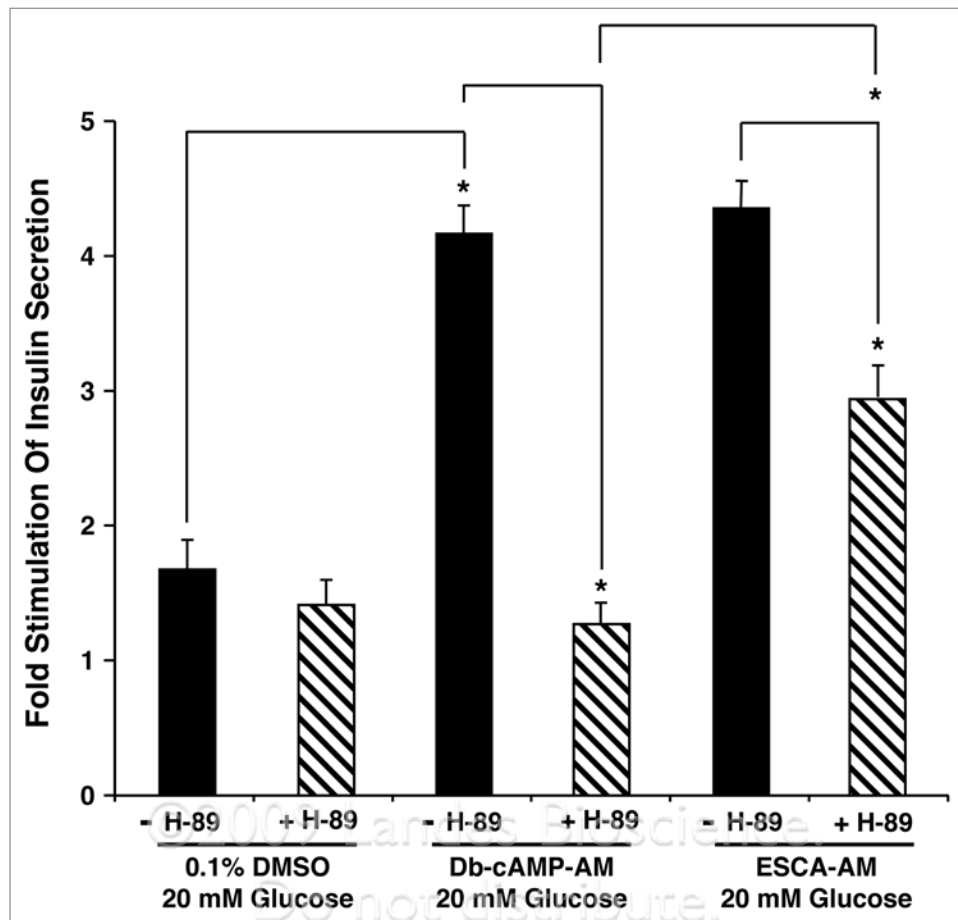
We have now expanded on these prior studies to demonstrate that 8-pCPT-2'-O-Me-cAMP-AM (10  $\mu$ M) potentiates the

stimulatory action of 20 mM glucose on mouse islet insulin secretion (Fig. 2). This action of 8-pCPT-2'-O-Me-cAMP-AM is not reproduced by 8-pCPT-2'-O-Me-cAMP nor is it mimicked by phosphate-AM<sub>3</sub> serving as a negative control (hydrolysis of the AM-ester liberates intracellular formaldehyde and acetic acid). Of primary significance is our finding that 8-pCPT-2'-O-Me-cAMP-AM potentiates both the first and second phases of GSIS, whereas it has no secretagogue action in the presence of a non-stimulatory concentration of glucose (Fig. 2). Thus, 8-pCPT-2'-O-Me-cAMP-AM recapitulates the glucose-dependent action of GLP-1 to stimulate islet insulin secretion.<sup>7-9</sup> Such effects of 8-pCPT-2'-O-

Me-cAMP-AM might be Epac2 mediated since this isoform of Epac is the predominant isoform expressed in mouse islets, as evaluated by quantitative PCR (QPCR) (Fig. 3).

One cautionary note for future users of 8-pCPT-2'-O-Me-cAMP-AM is that under conditions of long-term treatment, this ESCA will accumulate in  $\beta$ -cells so that its concentration might rise to levels high enough to activate PKA.<sup>27</sup> To address this issue, we sought to determine if 8-pCPT-2'-O-Me-cAMP-AM activated PKA in various insulin-secreting cell lines or in human  $\beta$ -cells. Our strategy was to expose these cells to 10  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM for 30 min, an approach identical to that used in our assays of islet insulin secretion. Using a Kemptide PepTag assay (Promega) in which the activated form of PKA was detected in cellular lysates, we demonstrated that 8-pCPT-2'-O-Me-cAMP-AM (10  $\mu$ M) failed to activate PKA in rat INS-1 cells, mouse MIN6 cells, and human islets (Chepurny OG, unpublished observations). Furthermore, we found that 8-pCPT-2'-O-Me-cAMP-AM failed to act in both INS-1 cells and MIN6 cells to promote Ser<sup>133</sup> phosphorylation of transcription factor CREB, a PKA substrate (Chepurny OG, unpublished observations).<sup>13</sup> Such findings concerning PKA and CREB are in accord with our prior report that 8-pCPT-2'-O-Me-cAMP-AM failed to stimulate the expression of a PKA-regulated luciferase reporter (RIP1-CRE-Luc) that incorporates a multimerized cAMP response element (CRE) found within the rat insulin 1 gene promoter (RIP1) and which we expressed in INS-1 cells.<sup>13,52,53</sup> Instead, we found that this reporter was stimulated by the selective PKA activator dibutyryl-cAMP-AM (Fig. 1).<sup>13</sup>

To expand on this analysis, we also performed FRET-based live-cell imaging assays to demonstrate that 8-pCPT-2'-O-Me-cAMP-AM (10  $\mu$ M) had only a marginal ability to activate a PKA biosensor (AKAR3) expressed by viral transduction of human  $\beta$ -cells. In contrast, the PKA activator 6-Bnz-cAMP-AM (10  $\mu$ M) produced an effect comparable to that of forskolin (Leech CA, Roe MW, unpublished observations). Furthermore, we recently succeeded in demonstrating that 8-pCPT-2'-O-Me-cAMP-AM (10  $\mu$ M) raised



**Figure 4.** PKA inhibitor H-89 fails to abrogate the action of 8-pCPT-2'-O-Me-cAMP-AM to potentiate GSIS in adult male C57BL/6J mouse islets. Illustrated are findings obtained in static incubation assays using methodologies of islet isolation and solution exposure described previously.<sup>13</sup> Islets were exposed to KRB containing 2.8 mM glucose for 30 min and were then exposed to KRB containing 20 mM glucose with or without added test substances. These test substances included H-89 (10  $\mu$ M), dibutyryl-cAMP-AM (Db-cAMP-AM; 10  $\mu$ M), and 8-pCPT-2'-O-Me-cAMP-AM (ESCA-AM; 10  $\mu$ M). Each test substance was dissolved in a vehicle solution comprised of KRB containing 0.1% DMSO and 0.1% BSA. The findings are representative of a single experiment repeated twice. Values of fold-stimulation were calculated by comparing insulin secretion measured in 20 mM glucose KRB relative to KRB containing 2.8 mM glucose. Values are mean  $\pm$  s.e.m. for duplicate determinations. Statistical significance was evaluated by the t-test (\* $p$  < 0.05).

levels of cytosolic  $Ca^{2+}$  in mouse  $\beta$ -cells and potentiated GSIS under conditions in which mouse islets were treated with H-89 (10  $\mu$ M) (Dzhura I, Dzhura E, unpublished observations). We found that treatment with H-89 reduced but did not block the action of 8-pCPT-2'-O-Me-cAMP-AM to potentiate GSIS, whereas H-89 completely abrogated the action of dibutyryl-cAMP-AM to potentiate GSIS (Fig. 4). These data are understandable if the Epac-mediated action of 8-pCPT-2'-O-Me-cAMP-AM to potentiate GSIS is contingent on some basal level of PKA activity that supports exocytosis. Alternatively, a 30 min exposure of mouse islets to a 10  $\mu$ M concentration of 8-pCPT-2'-O-Me-cAMP-AM might lead to full activation of Epac and partial activation of PKA.

If so, this source of activated PKA must not be detectable in the in vitro assays of PKA activity described above. Given such uncertainties, and in view of the fact that no selective antagonist of Epac activation exists, it is likely that future studies using Epac2 knockout mice will provide a better understanding of exactly how 8-pCPT-2'-O-Me-cAMP-AM interacts with  $\beta$ -cell glucose metabolism to stimulate islet insulin secretion.

In conclusion, the new information presented here demonstrate the remarkable efficacy of 8-pCPT-2'-O-Me-cAMP-AM as a potentiator of mouse islet GSIS. The challenge now is to extend upon this analysis and to establish what role if any Epac proteins play in the GPCR-mediated stimulation of islet insulin secretion. GPCR agonists

known to stimulate cAMP production and to potentiate GSIS include not only GLP-1 but also glucose-dependent insulinotropic peptide (GIP), pituitary adenylyl cyclase-activating peptide (PACAP), and oleylethanolamide (OEA, a GPR119 agonist).<sup>54</sup> Thus, it seems reasonable to speculate that the differential abilities of these agents to lower levels of blood glucose in type 2 diabetic subjects might be related to each agonist's propensity to signal predominantly through either Epac or PKA. In this vein, it is important to note that Epac1 is detectable in mouse islets and that the knockout of Epac1 was recently reported to interfere with glucose homeostasis in mice.<sup>55</sup> Thus, one important issue that remains outstanding concerns the potential dual roles Epac1 and Epac2 in  $\beta$ -cell function.

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