Epac is involved in cAMP-stimulated proglucagon expression and hormone production but not hormone secretion in pancreatic α- and intestinal L-cell lines

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Submitted 6 May 2008; accepted in final form 8 October 2008

The proglucagon gene (gcg) is expressed in the pancreatic islet α-cells, intestinal endocrine L cells, and selected neurons in the brain (4, 5, 11, 21, 30). The gcg mRNA encodes the prehormone proglucagon, which can be processed to produce three major peptide hormones: glucagon, glucagon-like peptide-1 (GLP-1), and glucagon-like peptide-2 (GLP-2; Ref. 6). Glucagon produced in the pancreatic islet α-cells is a major counterregulatory hormone to insulin in maintaining glucose homeostasis. In contrast, GLP-1 and GLP-2 are synthesized in and released by the intestinal endocrine L cells (11). GLP-1 has been shown to enhance glucose-dependent insulin secretion and inhibit glucagon release. Other physiological functions of GLP-1 include the stimulation of pancreatic β-cell growth and survival and the inhibition of gastric emptying (6). In addition, both peripheral and intracerebroventricular administration of GLP-1 inhibits food intake in fasted rodents (38, 44, 45). Furthermore, GLP-1 agonists are able to differentiate both somatic and embryonic stem cells into insulin-producing cells (1, 10, 32, 33, 49, 51). GLP-2 was originally demonstrated to serve as a growth factor of the small intestinal epithelium (3, 14, 46), while recent studies (16, 35, 48) have shown that it may exert some overlapping function with GLP-1 in controlling food intake.

Numerous studies (2, 15, 18, 19, 39, 47) have been conducted to explore the mechanisms underlying gcg expression, as well as production and secretion of glucagon and GLP-1. In both pancreatic α-cells and intestinal endocrine L cells, gcg transcription is enhanced in response to elevation of cAMP (12, 13, 15, 18, 39, 47). Secretion of glucagon and GLP-1 is also stimulated by activation of cAMP signaling (9, 15, 20, 37, 43). While precise mechanisms underlying these stimulatory effects have yet to be fully elucidated, recent studies have shown that in addition to activating PKA, cAMP can activate gcg transcription via other mechanisms (34, 39, 47). We (34) have recently reported that exchange protein directly activated by cAMP (EPAC) plays a role in stimulating gcg transcription in intestinal GLP-1-producing cell lines in response to cAMP elevation. Ma et al. (37) have, however, reported that Epac signaling is involved in glucagon secretion.

In the present study, using a dominant-negative strategy (Epac-2DN) and an Epac pathway-specific cAMP analog (ESCA), we further assessed the role of Epac signaling in the regulation of gcg expression, as well as glucagon and GLP-1 production and secretion in pancreatic α- and intestinal endocrine L-cell lines. Our findings suggest that, while Epac signaling plays a stimulatory role in the pancreatic and intestinal endocrine cells, including gcg transcription and glucagon and GLP-1 production, Epac is not involved in stimulating secretion of either glucagon or GLP-1 by these cultured endocrine cell lines.
MATERIALS AND METHODS

Reagents. Cell culture medium, FBS, horse serum, and calf serum were purchased from Invitrogen Life Technology (Burlington, Ontario, Canada). Forskolin and IBMX were purchased from Sigma Aldrich (Oakville, Ontario, Canada). The PKA inhibitor H89 was the product of Calbiochem (EMD Biosciences, San Diego, CA). An Epac pathway specific cAMP analog 8-(4-methoxyphenylthio)-2′-O-methyladenosine-3′,5′-cAMP (8-pMeOPT-2′-O-Me-cAMP), defined in this study as ESCA (23), was provided by BIOLOG Life Sciences Institute (Bremen, Germany).

Plasmids. The 2.4-kb rat proglucagon-luciferase (2.4-kb gcg-LUC) reporter plasmid was constructed as previously described (26). Mouse Epac-2 dominant-negative (Epac-2DN) plasmid was a gift from Dr. T. Shibasaki (29; Kobe University, Japan). The coding region of Epac-2DN was amplified with PCR and inserted into the pcDNA 3.1/myc-His expression vector (Invitrogen Life Technology). The primer sequences for this subcloning were as follows: forward primer, 5′-GGCGCAATTCCACATGGTCGTCGGCCGCAGCT-3′; and reverse primer, 5′-GGCGCGCCGCGCGCCTGAGCTGCTAA-3′.

Cell culture, DNA transfection, and LUC reporter gene analysis. Pancreatic islet α-cell lines InR1-G9, RIN1056A, and αTC-1; the pancreatic β-cell line INS-1; and intestinal gcg-producing STC-1 and GLUTag cell lines were maintained as previously described (15, 25, 34). The control fibroblast COS-1 cells were maintained in DMEM with 5% FBS. For LUC reporter gene analysis, cells were transiently transfected by calcium precipitation for InR1-G9 and GLUTag cell lines and by electroporation for the STC-1 cell line (25). Sixteen hours after transfection, cells were harvested and LUC reporter activity was measured as previously described (26). Data were normalized to the total protein utilized in the assay (26). InR1-G9 and STC-1 cells were stably transfected with myc-tagged Epac-2DN plasmid with lipofectamine for selection of stably clones per manufacturer’s instruction.

Northern blotting and RT-PCR. Total RNA was extracted using the TRizol reagent (Invitrogen Life Technology) according to the manufacturer’s instructions and was subjected to Northern blotting for gcg mRNA expression as described previously (25). Primers utilized in assessing the expression of Epac2, proglucagon, and proinsulin I in cultured cell lines are shown in Table 1.

Western blotting and immunohistochemistry. Expression of myc-tagged Epac-2 in transiently and stably transfected InR1-G9 and STC-1 cells was determined by Western blotting using the anti-Myc His expression vector (Invitrogen Life Technology). The primer sequences for this subcloning were as follows: forward primer, 5′-GGCGCAATTCCACATGGTCGTCGGCCGCAGCT-3′; and reverse primer, 5′-GGCGCGCCGCGCGCCTGAGCTGCTAA-3′.

RESULTS

Epac-2 is expressed in pancreatic α-cells. We (34) previously demonstrated that Epac-2 is expressed in the intestinal proglucagon-expressing GLUTag and STC-1 cell lines by Western blotting and immunostaining. To determine whether Epac-2 is also expressed in pancreatic α-cells, RT-PCR and immunostaining were performed. Figure 1A shows that Epac-2-specific primers amplified a fragment of the expected size (510 bp) from STC-1 and pancreatic β-INS-1 cells (positive controls; Ref. 34), as well as in three pancreatic gcg-producing α-cell lines, RIN-1056A, αTC-1, and InR1-G9. However, Epac-2 transcripts were not detected in the control COS-1 cells. DNA sequencing was performed to verify that the RT-PCR fragments obtained from the three pancreatic gcg-producing cell lines represent a portion of the Epac-2 cDNA (data not shown). We next determined the expression of Epac-2 at the protein level by immunostaining in the pancreatic InR1-G9 cell line. As shown in Fig. 1B, Epac-2 proteins were persistently detected in the InR1-G9 cells at an intensity that was comparable with that in the insulin-expressing INS-1 cell line, while no specific staining was detected in the negative control COS-1 cells. Taken together, our results indicate that Epac-2 is expressed in glucagon-expressing pancreatic α-cell lines at both the mRNA and protein levels.

ESCA stimulates gcg promoter and endogenous gcg expression in the PKA-deficient pancreatic InR1-G9 α-cell line. Utilizing ESCA, we assessed the effect of Epac activation on gcg mRNA expression in the InR1-G9 cell line. Figure 2A presents a representative Northern blotting result, showing that treatment of InR1-G9 cells with forskolin/IBMX (10 μM each) or ESCA (50 μM) for 4 h markedly increased gcg mRNA expression (comparing lane 1 vs. lane 2 and lane 3), while 10 μM ESCA had only a moderate stimulatory effect on gcg mRNA expression. The activation by forskolin/IBMX was only slightly attenuated by the PKA inhibitor H89 (comparing lane 2 vs. lane 3). Real-time RT-PCR was then utilized to quantitatively assess the effect of forskolin/IBMX and ESCA on gcg expression. As shown in Fig. 2B, forskolin/IBMX enhanced gcg mRNA expression by 2.7-fold, while 10 and 50 μM ESCA generated ~1.2- and 2.3-fold activation.

To investigate whether the activation of gcg expression by ESCA occurs at the transcriptional level, we treated 2.4-kb gcg-LUC-transfected InR1-G9 cells with different doses of ESCA for 4 h. As shown in Fig. 2C, while 2 or 10 μM ESCA had no noticeable effects on gcg promoter expression, 50 μM ESCA generated a 5.1-fold activation. A 7.2-fold response was seen with forskolin/IBMX (10 μM each) treatment. The relatively higher levels of activation of gcg promoter expression compared with those of endogenous gcg mRNA expression by cAMP promoting agents is likely due to the fact that in chromatin-free promoter-reporter gene assays the stimulatory effect cannot be attenuated as is the case with endogenous gene expression, as we have observed previously (25, 34).

Table 1. Primers utilized in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA Sequence</th>
<th>Product Size, bp</th>
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<tr>
<td>Epac-2F2</td>
<td>5′-GGCTTTTGGCTGCGCTGCTG-3′</td>
<td>251</td>
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<tr>
<td>Epac-2R2</td>
<td>5′-TTGACCTATCAGGGCAAGGTT-3′</td>
<td>160</td>
</tr>
<tr>
<td>Ins-1F</td>
<td>5′-GGCCGACACATGGACAGGAC-3′</td>
<td>510</td>
</tr>
<tr>
<td>Ins-1R</td>
<td>5′-AGCTGGCTCTGCGCTGCTGCTG-3′</td>
<td>160</td>
</tr>
<tr>
<td>Glu-1F</td>
<td>5′-AGTGAAGACTTTACTTTG-3′</td>
<td>510</td>
</tr>
<tr>
<td>Glu-1R</td>
<td>5′-GGCTTTTGGCTGCGCTGCTG-3′</td>
<td>254</td>
</tr>
</tbody>
</table>
Epac-2DN blocked cAMP-stimulated gcg promoter expression in the InR1-G9 cells. To further verify the role of Epac signaling in stimulating gcg expression in the intestinal L cells and to evaluate the role of Epac in the pancreatic \( \alpha \)-cells, we utilized a Epac-2DN construct in which the two cAMP-binding motifs are mutated (29). We first conducted cotransfection of three different dosages (1, 2, and 3 \( \mu \)g) of Epac-2DN or the empty cloning vector (pCDNA3.1) with a constant amount (2 \( \mu \)g) of 2.4-kb gcg-LUC reporter (26). As shown in Fig. 3A, in the PKA active intestinal STC-1 cell line, in the absence of Epac-2DN, forskolin/IBMX-stimulated gcg-LUC reporter expression was significantly reduced to 25-, 29-, and 26-fold, respectively, in the cells transfected with 1, 2, and 3 \( \mu \)g of Epac-2DN. The lack of complete inhibition of forskolin/IBMX-stimulated gcg-LUC reporter expression was 80-, 170-, and 40-fold, respectively, when 1, 2, and 3 \( \mu \)g of control (pCDNA3) plasmid were included.

Fig. 1. Detection of exchange protein directly activated by cAMP (Epac)-2 expression in pancreatic \( \alpha \)-cell lines. A: RT-PCR for the detection of Epac-2, proinsulin I (insulin), proglucagon (gcg), and actin mRNA transcripts in four pancreatic cell lines, the intestinal proglucagon-expressing STC-1 cell line (positive control), and the nonendocrine COS-1 cell line (negative control). B: immunohistochemistry and confocal microscopy for the detection of Epac-2 protein expression in INS-1, COS-1, and InR1-G9 cells. DIC, differential interference contrast (34).

Fig. 2. ESCA stimulates proglucagon mRNA and promoter expression in the InR1-G9 cell line. A: Northern blotting for proglucagon mRNA (gcg) transcripts in response to treatment with forskolin/IBMX or (Epac) pathway-specific cAMP analog (ESCA). rRNA, 18S, and 28S ribosomal RNAs. B: real-time RT-PCR for gcg mRNA expression in response to treatment with forskolin/IBMX and ESCA. Results are presented as relative gcg copy number with cells receiving no treatment as the control (means \( \pm \) SE; \( n = 3 \)). C: effect of forskolin/IBMX and ESCA on 2.4-kb gcg-LUC expression in the InR1-G9 cell line. Three micrograms of 2.4-kb gcg-LUC were transfected into the InR1-G9 cell line. Sixteen hours after transfection, cells were further treated with or without indicated reagents for another 4 h before being harvested for LUC reporter analysis. Relative LUC activity was calculated as fold induction with the activity in cells receiving no treatment set to 1-fold (means \( \pm \) SE; \( n = 3 \); ** \( P < 0.01 \); *** \( P < 0.001 \)).
Fig. 3. Epac-2DN transient transfection attenuates or blocks the stimulatory effect of forskolin/IBMX on gcg-LUC reporter expression in STC-1, GLUTag, and InR1-G9 cell lines. STC-1 (A), GLUTag (B), and InR1-G9 cells (C) were cotransfected with 2 μg of 2.4-kb gcg-LUC and indicated amount of pCDNA3.1 (control cloning vector) or Epac-2DN. Sixteen hours after transfection, cells were treated with forskolin/IBMX or vehicle for an additional 4 h before being harvested for LUC reporter analysis. Relative LUC activity was calculated as fold induction with the activity in cells receiving no treatment set to 1-fold (means ± SE; n is ≥3; *P < 0.05, **P < 0.01, ***P < 0.001).
IBMX-mediated activation by EpacDN expression would suggest that both PKA and Epac are involved in mediating the effect of cAMP elevation. We then conducted the same experiment in the PKA active intestinal GLUTag L-cell line. In the absence of Epac-2DN, forskolin/IBMX stimulated expression of the gcg-LUC reporter by 12.3-, 15.3-, and 11.5-fold, respectively, when 1, 2, and 3 µg of control (pCDNA3) plasmid were included (Fig. 3B). Epac2-DN cotransfection at the three different dosages significantly attenuated, but did not abolish, the stimulatory effect of forskolin/IBMX (Fig. 3B). Interestingly, we did not see a clear dose-dependent response to Epac-DN in either cell line, suggesting that in this system 2 µg of Epac-2DN were optimal to block the effect of Epac signaling.

In the PKA-deficient pancreatic InR1-G9 cell line, forskolin/IBMX treatment stimulated expression of the gcg-LUC reporter by 4.3- to 5.6-fold (P < 0.001; Fig. 3C). The relative lower level of activation by forskolin/IBMX in InR1-G9 may be due to the PKA-deficient nature of this cell line (7). Nonetheless, forskolin/IBMX-stimulated gcg-LUC expression was significantly repressed in the cells transfected with 1 µg Epac-2DN and was completely blocked in the InR1-G9 cells transfected with 2 or 3 µg Epac-2DN (Fig. 3C).

We then established both STC-1 and InR1-G9 cell lines that stably express Epac-2DN and assessed the effect of Epac-2DN expression on gcg promoter activity in two clones of the STC-1 cell line and six clones of the InR1-G9 cell line. A representative Western blot (Fig. 4A) demonstrates the expression of myc-tagged Epac-2DN in selected clones of these two cell lines. Figure 4B shows that forskolin/IBMX-stimulated gcg-LUC expression decreased from 105- to 85- and 58-fold (~27 and 48% reduction), respectively, in two of the Epac-2DN stably-transfected STC-1 clones. In the six InR1-G9 clones, Epac-2DN expression also significantly attenuated forskolin/IBMX-stimulated gcg-LUC expression (from 9.2- to 2.1- to 2.4-fold, or ~74–77% reduction). Figure 4C shows our analysis results of two of the six InR1-G9 clones. Taken together, these observations suggest that both PKA and Epac signaling are involved in cAMP-mediated activation of gcg transcription.

ESCA increases glucagon and GLP-1 production in InR1-G9 and GLUTag cell lines. Utilizing ESCA and forskolin/IBMX, we then assessed the effect of Epac activation on glucagon and GLP-1 production in the InR1-G9 and GLUTag cell lines by RIA. For this purpose, we determined the total amount of hormone produced by 3 × 10^5 cells in the presence and absence of a chemical treatment by measuring the total glucagon or GLP-1 content in the cell lysate plus the culture medium. In the InR1-G9 cells, 4-h treatment with 2, 10, and 50 µM ESCA increased total cellular glucagon levels by 21, 16, and 24%, respectively, while forskolin/IBMX treatment induced a 22% increase in glucagon production (Fig. 5A). In the GLUTag cell line, treatment of the cells with 50 µM ESCA for 0.25, 0.5, or 4 h generated a 26, 24, and 70% increase in GLP-1 levels, respectively, while forskolin/IBMX treatment for 4 h caused a 97% increase in GLP-1 production (Fig. 5B). No stimulation was observed in GLUTag cells treated with 10 µM ESCA for 4 h (data not shown).

ESCA does not stimulate glucagon and GLP-1 secretion in InR1-G9 and GLUTag cell lines. Finally, we assessed the effect of ESCA on glucagon and GLP-1 secretion in the two cultured cell lines by the determination of the hormone content of the culture medium by RIA. When ESCA was applied to either InR1-G9 or GLUTag cell lines, no notable increases in glucagon or GLP-1 secretion were observed, although forskolin/IBMX treatment was shown to enhance release of both peptides (Fig. 6, A and B). This observation would suggest that, despite similar increases in total hormone content (Fig. 5), forskolin/IBMX-mediated glucagon and GLP-1 secretion, respectively, may not be mediated via Epac signaling. Furthermore, consistent with the lack of active PKA in the InR1-G9 cells, forskolin/IBMX-mediated glucagon secretion was not inhibited by H89, suggesting the involvement of a PKA-independent pathway. Finally, consistent with the observations of Kang et al. (27), control studies demonstrated that 10 and 50 µM ESCA increased insulin secretion in the INS-1 cell line (Fig. 6C). Independent control studies demonstrated that the H89 utilized in this study was biologically active, as it did reduce forskolin/IBMX-induced peCRE-LUC reporter gene expression (see Supplemental Fig. 1; supplemental data for this
The stimulatory effect of cAMP signaling on proglucagon expression has been extensively studied in both pancreatic islet \( \alpha \)-cells and intestinal endocrine L cells (8, 12, 13, 15, 18, 31, 36, 41, 42, 47). Although a typical cAMP response element (CRE) is located between 291 bp and 298 bp of the rodent gcg promoter (31), a few studies (18, 36) have shown that deleting or mutating this element only moderately attenuated the stimulatory effect of cAMP elevation on proglucagon promoter expression. Furthermore, Fürstenau et al. (17) identified another motif within the G2 enhancer element (downstream of the typical CRE) that may mediate the stimulatory effects of both cAMP and calcium on gcg transcription. In studying the stimulatory effect of protein hydrolysates on proglucagon expression, Gevrey et al. (19) revealed the existence of two CRE-like elements (upstream of the typical CRE) that may mediate the stimulatory effects of both cAMP and amino acids. Additionally, the typical CRE motif is not conserved in the human gcg promoter (40), indicating that this motif may not be physiologically important (24). Collectively, extensive observations made by several laboratories suggest that elevations in cAMP levels lead to increased proglucagon transcription via multiple cis-elements within the proglucagon 

![Figure 5](http://ajpendo.physiology.org/)

**Fig. 5.** ESCA stimulates glucagon and glucagon-like peptide-1 (GLP-1) production. InR1-G9 (A) and GLUTag (B) cells were treated with indicated chemicals at indicated concentrations for 4 h. Glucagon (A) and GLP-1 (B) content in the medium plus cell lysates was measured by RIA. Results are presented as nanograms of hormone per 3 \( \times 10^5 \) cells (means \( \pm \) SE; \( n = \geq 3 \); \( *P < 0.05 \), \( **P < 0.001 \)).

![Figure 6](http://ajpendo.physiology.org/)

**Fig. 6.** ESCA does not stimulate glucagon or GLP-1 secretion. InR1-G9 (A), GLUTag (B), and INS-1 (C) cells were treated with indicated chemicals at indicated concentrations for 4 h. Glucagon (A), GLP-1 (B), and insulin (C) content in the medium was measured by RIA. Results are presented as nanograms of hormone per milliliters of medium, normalized for the number of cells. (means \( \pm \) SE; \( n = \geq 3 \); \( **P < 0.01 \), \( ***P < 0.001 \)).
and block the effects of forskolin/IBMX, respectively. These observations collectively suggest that Epac plays an important role in mediating the stimulatory effect of cAMP on proglucagon transcription.

How Epac activation leads to enhanced gcg transcription is not fully understood. Nevertheless, Epac signaling could be involved in regulating the expression and function of transcriptional activators of gcg, such as the homeodomain protein Cdx-2 (7). We (7) have shown previously that the expression of Cdx-2 can be activated by Epac activation, possibly by the utilization of MEK-ERK1/2 as the effector. Furthermore, Epac is known to enhance intracellular calcium levels (28, 29), a known effector of proglucagon promoter expression in the pancreatic αTC cell line (17).

Although no notable stimulatory effect of 10 μM ESCA on the gcg gene promoter expression was observed in the PKA-deficient InR1-G9 cell line (Fig. 2C), the same concentration of ESCA did slightly enhance the total content of the glucagon peptide (Fig. 5A). This raises the question as to whether the Epac pathway regulates glucagon production by more than one mechanism, including one that does not involve gcg transcription. In the PKA-active GLUTag cell line, the stimulatory effects of both ESCA and forskolin/IBMX on total GLP-1 content were considerable higher than those observed for glucagon in the InR1-G9 cell line, suggesting that both PKA and Epac may be involved in mediating the stimulatory effects of cAMP elevation on the production of gcg-encoded peptide hormones.

Interestingly, although ESCA was shown to stimulate insulin secretion from the INS-1 cell line, as demonstrated in the present study and by Kang et al. (28), we did not observe a notable effect on release of glucagon from the hamster InR1-G9 cell line or of GLP-1 from the murine GLUTag cell line. This is in contrast with a previous study (28, 37) in rat primary pancreatic islet cells in which a substantially higher concentration of ESCA (100 μM) was found to enhance glucagon release from primary rat islets. These differential responses may be attributable to species and/or dose-dependent differences between the two studies. In addition, paracrine regulation by adjacent non-α-cells could directly influence glucagon secretion by isolated islets (20).

In summary, we report here that the Epac pathway stimulates the production but not the release of glucagon and GLP-1 in cultured endocrine cell lines. In contrast, activation of PKA enhanced both the production and secretion of these peptide hormones. Epac signaling may therefore provide a novel approach to enhance cellular levels of glucagon and GLP-1, in preparation for subsequent release into the circulation, without actually affecting whole body glucose homeostasis.

ACKNOWLEDGMENTS

We thank Dr. Tadao Shibasaki for providing the original Epac-2DN expression plasmid and Dr. Dingyan Wang for technical assistance.

GRANTS

This study was supported by operating grants from the Canadian Diabetes Association to T. Jin (2341) and P. L. Brubaker (2374), the Canadian Institute of Health Research to Q. Wang (79534), and Juvenile Diabetes Research Foundation to H. Y. Gaisano (1-2009-1112). P. L. Brubaker is supported by the Canada Research Chairs Program. Q. Wang is the recipient of a New Investigator Award from the Canadian Institute of Health Research, and D. Islam is the recipient of a Banting and Best Diabetes Centre–Novo Nordisk Studentship.

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