cAMP Dose-dependently Prevents Palmitate-induced Apoptosis by Both Protein Kinase A- and cAMP-Guanine Nucleotide Exchange Factor-dependent Pathways in β-Cells*

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Lipid accumulation in pancreatic β-cells is thought to cause its dysfunction and/or destruction via apoptosis. Our studies show that incubation of the β-cell line RINm5F with the saturated free fatty acids (FFA) palmitate caused apoptosis based on increases in caspase 3 activity, Annexin V staining, and cell death. Furthermore, exposure of RINm5F cells to cAMP-increasing agents, 3-isobutyl-1-methylxanthine (IBMX), and forskolin completely abolished palmitate-mediated caspase 3 activity and significantly inhibited Annexin V staining and cell death. The cyclic AMP analogs cpt-cAMP and dibutyryl-cAMP mimicked the protective effects of IBMX and forskolin, suggesting that cAMP is the mediator of the anti-apoptotic effects. The protective action of IBMX and forskolin was rapid and did not appear to require gene transcription or new protein synthesis. However, these protective effects were clearly independent of protein kinase A (PKA) because of the lack of inhibition by the PKA inhibitors H-89 and KT5720. In attempts to identify this PKA-independent mechanism, we found that the newly developed cAMP analog 8CPT-2Me-cAMP, which selectively activates the cAMP-dependent guanine nucleotide exchange factor (cAMP-GEF) pathway, mimicked the protective effects of IBMX and forskolin, suggesting that the cAMP-GEF pathway is involved. In addition, both glucagon-like peptide (GLP-1) and its receptor agonist, Exenatide, inhibited palmitate-mediated caspase 3 activation in a dose-dependent manner. Unexpectedly, H-89 partially reversed the protective effects of GLP-1 and Exenatide, suggesting that PKA may play a role in the protective effects of these incretins. To explain these seemingly conflicting results, we demonstrated that low concentrations of cAMP produced by GLP-1 and Exenatide preferentially activate the PKA pathway, whereas higher cAMP concentrations provided by IBMX and forskolin activate the more dominant cAMP-GEF pathway. Taken together, these results indicate that intracellular concentrations of cAMP may play a key role in determining divergent signaling pathways that lead to antiapoptotic responses.

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‡ Obesity is a leading factor associated with the development of type 2 diabetes. Elevated levels of FFA in the circulation of obese subjects have been postulated to cause peripheral and hepatic insulin resistance and impairment of insulin secretion from pancreatic β-cells (1). Studies in Zucker diabetic fatty (ZDF) rats have indicated that chronically elevated levels of FFA cause lipid accumulation in β-cells and lead to β-cell dysfunction and destruction via apoptosis (2).

Elevated levels of intracellular cAMP have been reported to protect various cell types including neutrophils, hepatocytes, and neuronal cells from apoptosis stimulated by various agents (3–6). The mechanisms responsible for the protective action of cAMP against apoptosis include the synthesis of antiapoptotic proteins, inactivation of pro-apoptotic proteins, and activation of phosphatidylinositol 3-kinase-dependent Akt. Interestingly, cAMP has been also shown to promote apoptosis in leukemia and glioma cells by abnormal cell cycle regulation (7, 8). Therefore, it appears that cAMP can cause both apoptotic and anti-apoptotic effects depending on the cell type and triggering stimulus. The antiapoptotic effects of cAMP on lipo toxicity, however, have not been previously reported.

The activation of protein kinase A (PKA) by cAMP is a classical cellular signaling pathway that regulates a variety of cellular responses. The specificity of this regulation by cAMP is provided by protein kinase A-anchoring proteins that target its receptor, PKA, to specific cellular locations (9). It has been shown that mitochondria-anchored PKA inactivates the pro-apoptotic factor BAD through phosphorylation in response to the survival cytokine interleukin-3. Interleukin-3-induced phosphorylation of BAD was blocked by the PKA inhibitors Rp-cAMP and H-89 consistent with anchored PKA as the principal kinase (9).

Recent studies indicate that cAMP causes an even broader range of cellular responses by activating signaling pathways that are independent of PKA. Cyclic AMP has been shown to regulate gene transcription, cellular proliferation, and cytokine signaling through PKA-independent pathways (10–13). A novel PKA-independent pathway that is activated by cAMP involves the guanine nucleotide exchange factors (GEFs also designated as Epac) for the small GTPases Rap1 and Rap2. It

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has been demonstrated that cAMP-GEFs directly activate Rap1, which is clearly independent of PKA (14, 15). However, the functional consequences of the activation of Rap1 by the cAMP-GEF pathway are not fully understood. The recent development of a novel cAMP analog, 8-(chloro-phenylthio)-2'-O-methyl-adenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP), which activates cAMP-GEF, but not PKA, has been used to identify the cAMP-GEF signaling pathway (16). Furthermore, two isoforms of GEF have been identified, GEF-1 and GEF-2, and pancreatic islets and β-cell lines express both isoforms (17). The cAMP-GEF pathway has been previously shown to regulate incretin-potentiated insulin secretion (18).

It is postulated that cAMP binds to GEF, and the cAMP-GEF complex activates Rap1, a ubiquitously expressed GTPase of the Ras superfamily. Both Ras and Rap1 can exist in an inactive GDP-bound form or an active GTP-bound form, and cAMP-GEF promotes the formation of the active GTP-bound form of GTPase. Although the precise function of Rap1 is not known, it is believed to exhibit both Ras-dependent and -independent cellular functions. Recent studies suggest that the Ras/mitogen-activated protein kinase proliferation signaling pathway may regulate GLP-1-mediated β-cell growth and proliferation (17). Recent evidence also indicates that Ca2+-induced Ca2+ release is regulated by the cAMP-GEF pathway in the β-cell line, INS-1 (19), and the ability of incretins to potentiate glucose-stimulated insulin secretion from isolated mouse islets involves both a PKA-dependent pathway and a PKA-independent pathway. The PKA-independent pathway involves the interaction of GEF-2 and Rim2, an insulin granule-associated protein (18).

Glucagon-like peptide-1 (GLP-1) is a 30-amino acid peptide secreted from L-cells of the intestinal epithelium in response to food (20). GLP-1 is one of the most potent insulinotropic substances known with half-maximal effective concentrations around 10 pmol/liter (21) and a strict requirement for glucose concentrations above 4.5 mmol/liter. GLP-1 mediates its effects by binding to cell surface receptors coupled to heterotrimeric G proteins, which are expressed by pancreatic β-cells. The binding of GLP-1 to its receptor stimulates CAMP formation, activation of PKA-dependent and -independent pathways, and a rise in intracellular Ca2+ concentrations (22, 23). GLP-1 has been shown to be effective in normalizing fasting glucose concentrations of type 2 diabetic patients resistant to diet and sulfonylurea therapy (24). GLP-1 has also been shown to protect β-cells from glucose toxicity in vitro by increasing islet cell proliferation and decreasing cellular apoptosis (25). The extensive and rapid degradation of GLP-1 after subcutaneous injection, however, limits its usage as a therapeutic agent. A number of strategies to prolong the half-life of GLP-1 are currently being explored. The GLP-1 receptor agonist, Exenatide, exhibits a longer half-life than GLP-1 because of its resistance to the ubiquitously expressed enzyme, dipeptidyl peptidase IV.

In this study, we have developed an in vitro model of palmitate-induced apoptosis of the β-cell line RINm5F and demonstrated that cAMP-increasing agents including IBMX and forskolin and the G protein-coupled receptor agonists GLP-1 and Enexatide provide protection in both a PKA- and cAMP-GEF-dependent manner. Low concentrations of cAMP produced by GLP-1 and Exenatide preferentially activate the PKA pathway, whereas higher cAMP concentrations produced by IBMX and forskolin activate the more dominant cAMP-GEF pathway. These findings suggest that modulation of cAMP concentrations may provide a strategy to optimize antiapoptotic signaling pathways.
Cell Viability Assays—After experimental treatment, both floating and attached cells were harvested and transferred to microcentrifuge tubes, and cell viability assays were performed. The cells were counted using a hemacytometer (Hauser Scientific, Horsham, PA) after mixing with trypan blue in a 1:1 ratio.

Assay of Phosphorylated and Total cAMP Response Element-binding Protein (CREB)—The cells (3 × 10⁵) were plated and cultured overnight in 35-mm Petri dishes. The cells were then pretreated for 30 min with H-89 (10 μM), followed by stimulation with forskolin (10 μM) for 10 min. The cells were washed once in 1× phosphate-buffered solution, solubilized with boiling SDS sample buffer, subjected to electrophoresis, and transferred to nitrocellulose. Anti-phosphorylated CREB and anti-CREB antibodies (Cell Signaling Technology, Beverly, MA) were used following the manufacturer’s recommendations and detected by chemiluminescence using an horseradish peroxidase-conjugated donkey anti-rabbit antibody.

cAMP Assays—Cells (3 × 10⁵) were plated in a 24-well plate. After experimental treatment, incubation media were aspirated and placed in microcentrifuge tubes. The cells were lysed by the addition of 500 μl of 0.1 N HCl containing 0.1% Triton X-100 and incubated for 10 min at room temperature. The lysed cells were scraped into microcentrifuge tubes. The samples were centrifuged at 1300 × g for 5 min at room temperature. Cyclic AMP concentrations in these supernatants were determined with a direct enzyme immunoassay kit as described by the manufacturer. Cyclic AMP concentrations in the culture media were also determined. The total concentration of cAMP produced by each condition was obtained by adding cellular and culture media cAMP contents.

Statistics—Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences were evaluated using Tukey post hoc analysis.

RESULTS

Cyclic AMP Protects RINm5F Cells from Palmitate-mediated Apoptosis—In this in vitro model, we have demonstrated that incubation of the β-cell line RINm5F with the saturated FFA palmitate causes an increase in apoptosis based on caspase 3 activity, Annexin V staining, and assays of cell death. The concentration dependence of this effect indicated that 300 μM palmitate complexed with BSA in a ratio of 6.6:1 (FFA:BSA) for 12 h caused an optimal increase in the number of β-cells undergoing apoptosis (data not shown). Under these experimental conditions, the free or unbound FFA concentration is estimated to be in the several hundred nanomolar range (26).

As shown in Fig. 1A, incubation of RINm5F cells with palmitate and the cAMP-increasing agents IBMX (100 μM) and forskolin (10 μM) completely prevented palmitate-mediated increases in caspase 3 activity. In a similar manner, IBMX and forskolin also significantly prevented, albeit to a lesser degree, palmitate-mediated increases in Annexin V staining and cell death as shown in Fig. 1 (B and C). The cyclic AMP analogs, chlorophenylthio-cAMP (cpt-cAMP; 200 μM) or dibutyryl-cAMP (1 mM) mimicked the effects of IBMX and forskolin, suggesting that the action of IBMX and forskolin is mediated by increases in intracellular cAMP (Fig. 2). Direct measurements of cAMP concentrations under these identical conditions have also been performed as shown in Table I. IBMX and forskolin stimulated cAMP production ~100-fold higher than control and palmitate conditions. The specific concentrations of IBMX, forskolin, and the cyclic AMP analogs employed in these experiments were selected based on dose-response studies (data not shown).

The time required for cAMP-increasing agents to prevent palmitate-mediated increases in caspase 3 activity was next evaluated. As shown in Fig. 3, palmitate-mediated increases in caspase 3 activity were completely prevented by exposure of RINm5F cells to cAMP-increasing agents present for the duration of a 12-h incubation period. The addition of IBMX and forskolin to the culture media 1 h prior to the end of a 12-h incubation with palmitate was sufficient to cause a significant decrease in caspase 3 activity. These findings suggest that the effects of IBMX and forskolin are rapid and probably do not require gene transcription or new protein synthesis. These cAMP-increasing agents were also added after the 12-h incubation period with palmitate and processed for caspase 3 activity. This condition did not result in inhibition of palmitate-
mediated apoptosis, suggesting that IBMX and forskolin did not interfere with the measurement of caspase 3 activity.

The Protective Effects of IBMX and Forskolin Are Dependent on cAMP-GEF and Independent of PKA—The involvement of PKA activation by cAMP was studied using two PKA inhibitors, H-89 and KT5720. As shown in Fig. 4 (A and B), various doses of H-89 (1–50 μM) or KT5720 (0.1–10 μM) did not reverse the inhibitory effects of IBMX and forskolin on palmitate-mediated caspase 3 activity. In the absence of either palmitate or IBMX + forskolin, neither H-89 nor KT5720 affected basal levels of caspase 3 activity (Fig. 4, white columns). To show that H-89 was able to inhibit PKA in intact cells, its ability to block cAMP-stimulated phosphorylation of CREB was examined. As shown in Fig. 5, H-89 (10 μM) was able to inhibit forskolin-stimulated phosphorylation of CREB to basal levels. These studies suggest that the protective effects of these cAMP-increasing agents are independent of PKA activation.

Recent studies have shown that CAMP directly activates the guanine nucleotide exchange factor (cAMP-GEF) pathway in a PKA-independent manner (14). The novel cAMP agonist, 8CPT-2Me-cAMP, designed to selectively activate the cAMP-GEF pathway but not PKA, was used to determine whether this signaling pathway is involved in the protective effects of IBMX and forskolin on palmitate-mediated apoptosis. Fig. 6 shows that 8CPT-2Me-cAMP (10 and 100 μM) completely prevented palmitate-mediated caspase 3 activity, suggesting that the cAMP-GEF pathway may be involved in the protective action of IBMX and forskolin on palmitate-mediated apoptosis.

GLP-1 and Its Receptor Agonist, Exenatide, Prevent Palmitate-mediated Apoptosis—GLP-1 has been shown to decrease β-cell apoptosis in ZDF rats (20). Based on our studies indicating that CAMP prevents palmitate-mediated apoptosis, we determined next whether the ability of GLP-1 to decrease apoptosis in ZDF islets may be due, in part, to increases in CAMP levels that directly regulate the cAMP-GEF pathway. RINm5F

Table I

| cAMP production by RINm5F cells | pmol·6 × 10^6 cells |
|---------------------------------|---------------------|
| Control                         | 4.3 ± 1.2           |
| Palmitate (300 μM)              | 10.5 ± 4.6          |
| Palmitate + IBMX (100 μM) + forskolin | 1326.5 ± 305.1 |

Fig. 3. Time requirements of IBMX + forskolin incubation on palmitate-mediated apoptosis. RINm5F cells (3 × 10^6) were treated with control medium (first column), medium containing 300 μM palmitate (second column), or medium in the presence of palmitate + IBMX (100 μM) + forskolin (10 μM) (third column) for 12 h. To determine the time requirements for the protective effects of IBMX + forskolin incubation on palmitate-mediated apoptosis, the cells were treated with medium containing 300 μM palmitate for 12 h, but IBMX + forskolin were present only during the final hour of the 12-h incubation (fourth column). As a control to verify that IBMX + forskolin did not interfere with the caspase 3 activity assay, these agents were added after the 12-h incubation (fifth column), and the cells were assayed for caspase 3 activity. The results are representative of three individual experiments.

Fig. 4. IBMX + forskolin protect RINm5F cells from apoptosis in a PKA-independent manner. RINm5F cells (3 × 10^6) were incubated with control medium or medium containing 300 μM palmitate, or palmitate + IBMX (100 μM) + forskolin (10 μM) for 12 h as previously described. To test the effects of PKA inhibitors on the protective effects of IBMX + forskolin on palmitate-mediated caspase 3 activity, the cells were treated with various concentrations of H-89 (A) or KT5720 (B), in the presence of palmitate + IBMX + forskolin for 12 h as indicated by the black bars. The cells were also incubated with various concentrations of PKA inhibitors alone as indicated by the white bars. Caspase 3 assays were then performed. The results are representative of three individual experiments. Pal, palmitate; For, forskolin; KT, KT5720.
cells were treated with palmitate and increasing concentrations of GLP-1 or its receptor agonist, Exenatide. As shown in Fig. 7, both GLP-1 and Exenatide provided significant protection from palmitate-mediated caspase 3 activation. This protection by GLP-1 and Exenatide occurred in a concentration-dependent manner and maximally reduced the caspase 3 activity to ~65% of its activity in the presence of palmitate alone. GLP-1 or Exenatide alone did not alter basal caspase activity as indicated by the white columns. Experiments were next performed to determine whether the ability of GLP-1 or Exenatide to provide protection against palmitate-induced apoptosis is due to activation of the cAMP-GEF pathway similar to the effects of IBMX and forskolin. Unexpectedly, an inhibitor of PKA, H-89 (1 and 10 μM), almost completely reversed GLP-1-mediated (1 μM) and Exenatide-mediated (100 nM) protection against apoptosis as shown in Fig. 8. To understand these seemingly conflicting results, we hypothesized that cAMP may activate both PKA and cAMP-GEF pathways depending on its concentration. To test this hypothesis, RINm5F cells were incubated with two different concentrations of forskolin (1 and 10 μM) or Exenatide, in the presence of palmitate, to determine whether these treatments could invoke different concentrations of intracellular cAMP. Fig. 9A indicates that 1 and 10 μM forskolin (third and fourth columns) indeed resulted in dose-dependent increases in cAMP. Moreover, Exenatide (fifth column) and GLP-1 (data not shown) stimulated cAMP production to a level similar to 1 μM forskolin. As shown in Fig. 9B (fourth and sixth columns), forskolin at 1 and 10 μM also resulted in a concentration-dependent protection of RINm5F cells against palmitate-mediated apoptosis. In addition, 1 μM forskolin-mediated protection was readily reversed by H-89 (Fig. 9B, fifth column), whereas 10 μM forskolin-mediated protection was not (Fig. 9B, seventh column), suggesting that cAMP activates these different signaling pathways in a concentration-dependent manner. Thus, lower concentrations of cAMP produced by 1 μM forskolin stimulate the PKA pathway, which is reversed by H-89, whereas the higher concentrations of cAMP produced by 10 μM forskolin stimulate the cAMP-GEF pathway, which is PKA-independent. The dose-dependent activation by cAMP of two different cellular signaling pathways has been confirmed using Exenatide in the absence or presence of a phosphodiesterase inhibitor, IBMX. As shown in Fig. 9A, Exenatide (100 nM) stimulated cAMP production at a level similar to forskolin (1 μM). The presence of IBMX in addition to Exenatide increases intracellular cAMP levels about 2-fold compared with Exenatide alone (data not shown). Although cAMP levels stimulated by Exenatide as shown in Fig. 9A are not as high as those stimulated by forskolin (10 μM), these lower levels appear to be sufficient to further enhance the protective effects of Exenatide against apoptosis as shown in Fig. 10A (fifth column). In Fig. 10A, IBMX (sixth column) or Exenatide (fourth column) alone protected palmitate-mediated apoptosis ~25–30%. However, when these agents were combined (fifth column), the protection was further enhanced to ~75%. To further support our findings that different cAMP levels activate alternative downstream signaling pathways, studies were performed with Exenatide as described in Fig. 10A but in
the presence of the PKA inhibitor, H-89. As shown in Fig. 10B, H-89 (10 μM) readily reversed Exenatide-mediated protection (fourth and fifth columns) against palmitate-induced apoptosis. In contrast, a combination of Exenatide and IBMX (sixth and seventh columns) that results in increased cAMP levels produced almost complete protection that was not reversed by H-89.

**DISCUSSION**

Our studies have demonstrated that palmitic acid induces apoptosis in the β-cell line RINm5F, and intracellular concentrations of cAMP exert a key role in modulating antiapoptotic responses. In this in vitro design, activation of caspase 3 activity provided greater sensitivity in assessing palmitate-mediated apoptosis than Annexin V or cell viability based on trypan blue staining.

In the course of these studies, we observed that the cAMP-increasing agents IBMX and forskolin completely prevented palmitate-mediated apoptosis of the β-cell line RINm5F. The protective action of IBMX and forskolin was rapid and did not require prolonged incubations, suggesting that gene transcription or new protein synthesis was not involved. Although increases in cAMP concentrations augment nutrient stimulation of insulin secretion from β-cells, alterations in insulin concentrations in the incubation media failed to affect palmitate-mediated apoptosis (data not shown). Furthermore, different concentrations of glucose did not alter the protective effects of cAMP against palmitate-mediated apoptosis (data not shown). Separate experiments demonstrated that forskolin alone was almost as effective as the combination of IBMX and forskolin, and IBMX alone provided an intermediate level of protection based on this same comparison. The asterisk denotes a statistically significant difference (p < 0.001) between the indicated columns. Pal, palmitate; For, forskolin.
ability of these two structurally unrelated PKA inhibitors to reverse palmitate-mediated apoptosis suggested that this protective action of cAMP occurred in a PKA-independent manner.

Interestingly, de Rooij et al. (14) reported that forskolin and cAMP activate Rap1, a small Ras-like GTPase, also in a PKA-independent manner. These investigators determined that cAMP directly activates a GEF (also designated as Epac) that in turn activates Rap1 in Chinese hamster ovary cells. More recently, Kang et al. (28) reported that cAMP triggers Ca^{2+}-induced Ca^{2+} release in pancreatic β-cells through activation of cAMP-GEF in a PKA-independent manner. These findings were accomplished, in part, by the use of a newly developed cAMP analog, 8CPT-2Me-cAMP, that selectively activates the cAMP-GEF pathway (16). Our studies indicate that this selective cAMP analog for the cAMP-GEF pathway mimicked the ability of cAMP-increasing agents to provide complete protection from palmitate-induced apoptosis. This protection by 8CPT-2Me-cAMP also occurred over a similar concentration range in which 8CPT-2Me-cAMP did not activate PKA signaling mechanisms involving CREB in the β-cell line, INS-1, as reported by Kang et al. (28).

Recent studies have also indicated that the cAMP-GEF signaling pathway exerts a critical role in GLP-1-potentiated insulin secretion by β-cells (29). In addition to its insulinotropic effects, GLP-1 has also been reported to promote islet cell growth and to inhibit apoptosis in ZDF rats (20). Our studies have shown that GLP-1 and its receptor agonist, Exenatide, also provided a significant degree of protection in a concentration-dependent manner against palmitate-mediated apoptosis of the β-cell line, RINm5F (Fig. 6). The lack of complete protection in comparison with IBMX and forskolin (10 μM) is believed to be due to the reduced concentrations of cAMP produced by these incretins (Fig. 8A). Paradoxically, the protection provided by GLP-1 or Exenatide against palmitate-mediated apoptosis was almost completely reversed by H-89, suggesting that a PKA-dependent pathway is involved in the protective actions of incretins unlike those of forskolin. A possible mechanism to explain these divergent findings is that the intracellular concentrations of cAMP generated by GLP-1 or Exenatide as described in these studies are postulated to activate the PKA pathway, whereas high concentrations of cAMP generated by 10 μM forskolin or 8CPT-2Me-cAMP activate the more dominant cAMP-GEF pathway.

A postulated mechanism to explain the antiapoptotic actions of GLP-1, Exenatide, and forskolin as described in these studies is shown in Fig. 11. The interaction of GLP-1 or Exenatide...
with its receptor activates heterotrimeric $G_{\alpha}$ proteins, leading to the dissociation of the $G_{\beta\gamma}$ subunit that subsequently stimulates adenyl cyclase to produce cAMP. Cyclic AMP produced by this $G_{\alpha}$-coupled receptor mechanism or by forskolin can activate either the PKA and/or cAMP-GEF pathway depending on its intracellular concentration. The specific downstream signaling mediators of the PKA or the cAMP-GEF pathway that are responsible for these antiapoptotic effects are currently unknown. An elucidation of this cellular mechanism and its relevance to FFA-induced apoptosis in primary $\beta$-cells is of importance to optimize conditions for $\beta$-cell growth and proliferation with minimal apoptosis.

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