Ca^{2+}-induced Ca^{2+} Release via Inositol 1,4,5-trisphosphate Receptors Is Amplified by Protein Kinase A and Triggers Exocytosis in Pancreatic β-Cells*

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Hormones, such as glucagon and glucagon-like peptide-1, potently amplify nutrient stimulated insulin secretion by raising cAMP. We have studied how cAMP affects Ca^{2+}-induced Ca^{2+} release (CICR) in pancreatic β-cells from mice and rats and the role of CICR in secretion. CICR was observed as pronounced Ca^{2+} spikes on top of glucose- or depolarization-dependent rise of the cytoplasmic Ca^{2+} concentration ([Ca^{2+}]_i). cAMP-elevating agents strongly promoted CICR. This effect involved sensitization of the receptors underlying CICR, because many cells exhibited the characteristic Ca^{2+} spiking at low or even in the absence of depolarization-dependent elevation of [Ca^{2+}]_i. The cAMP effect was mimicked by a specific activator of protein kinase A in cells unresponsive to activators of cAMP-regulated guanine nucleotide exchange factor. Ryanodine pretreatment, which abolishes CICR mediated by ryanodine receptors, did not prevent CICR. Moreover, a high concentration of caffeine, known to activate ryanodine receptors independently of Ca^{2+}, failed to mobilize intracellular Ca^{2+}. On the contrary, a high caffeine concentration abolished CICR by interfering with inositol 1,4,5-trisphosphate receptors (IP_3Rs). Therefore, the cell-permeable IP_3 antagonist 2-aminoethoxydiphenyl borate blocked the cAMP-promoted CICR. Individual CICR events in pancreatic β-cells were followed by [Ca^{2+}]_i spikes in neighboring human erythrocytoma cells, used to report secretory events in the β-cells. The results indicate that protein kinase A-mediated promotion of CICR via IP_3Rs is part of the mechanism by which cAMP amplifies insulin release.

Glucose is the most important physiological stimulator of insulin secretion from pancreatic β-cells. A major signal transduction pathway involves metabolism of glucose with increase of the ATP/ADP ratio, depolarization caused by closure of ATP/ADP-sensitive K⁺ (K_ATP) channels, and opening of L-type Ca^{2+} channels with influx of the ion. The resulting elevation of the cytoplasmic Ca^{2+} concentration ([Ca^{2+}]_i) triggers exocytosis of the insulin-containing granules (1). By stimulating Ca^{2+} sequestration in the endoplasmic reticulum (ER) (2–4), glucose also has an important role in preparing the β-cell to respond to hormones and neurotransmitters, which act by mobilizing Ca^{2+} from the ER (5–7). The latter effects are in most cases caused by activation of phospholipase C, catalyzing the formation of inositol 1,4,5-trisphosphate (IP_3). The IP_3 receptor (IP_3R) is a Ca^{2+} channel in the ER membrane (8). Another putative pathway for Ca^{2+} release from the ER is via ryanodine receptors (RyRs). Although RyRs are expressed in β-cells (9–12), their physiological role remains controversial (12–14). Ca^{2+}-induced Ca^{2+} release (CICR) is a mechanism by which any local rise of [Ca^{2+}]_i, becomes further amplified by Ca^{2+} release from stores. The heart is a classic example of CICR, where it provides a link between depolarization-dependent influx of “triggering” Ca^{2+} and release of contraction-inducing Ca^{2+} from the sarcoplasmatic reticulum (15). In heart cells, CICR is caused by activation of RyRs. However, in many other types of cells, IP_3Rs are equally competent in mediating CICR because they display a similar autocatalytic Ca^{2+} release mechanism (16). The binding of IP_3 thus sensitizes the IP_3Rs to the stimulatory effect of Ca^{2+} (17, 18).

As in the heart, CICR in the β-cell may provide a link between influx of Ca^{2+} and release from intracellular stores, resulting in amplification of the Ca^{2+} signal triggering insulin secretion (19). Several studies propose that CICR in β-cells is mediated by RyRs (10, 19–22). Critical experiments in the latter studies rely on the use of tumor-transformed clonal β-cells, and we have recently confirmed the expression of functional RyRs in rat insulinoma cells (23). However, our study also showed that CICR in primary β-cells from mice, rats, and human subjects is caused by activation of IP_3Rs rather than RyRs. Agents raising cAMP have been found to promote intracellular Ca^{2+} mobilization in insulin-releasing cell lines and pancreatic β-cells, and this action was proposed to be mediated by sensitization of either IP_3Rs (24–26) or RyRs (9, 10, 20–22, 26, 27). Phosphorylation of the RyRs by the cAMP-dependent protein kinase A (PKA) was assumed to be a prerequisite for CICR (9, 10). However, in later studies, a PKA-independent pathway involving cAMP-regulated guanine nucleotide ex-
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change factor (Epac) has been suggested (20, 22, 27). Because important evidence for this concept was obtained with cloned β-cells, we now have studied the mechanisms by which cAMP promotes CICR in primary mouse and rat β-cells. We show that CAMP-facilitated CICR is caused by PKA-dependent activation of IP\(_3\)R. Moreover, our data indicate that CICR is part of the mechanism by which CAMP amplifies insulin release.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents of analytical grade and deionized water were used. Fura-2 and its acetoxyethyl ester (fura-2/AM), fluo-4 acetoxyethyl ester (fluo-4/AM), and rhodamine were from Molecular Probes Inc. (Eugene, OR). Biolog Life Science Institute (Bremen, Germany) was the source of 8-(4-chlorophenylthio)-cGMP, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT-2′-O-Me-cAMP), 8-(4-methoxyphenylthio)-2′-O-methyl-cAMP (8-pMeOPT-2′-O-Me-cAMP) as well as the Sp and Rp isomers of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate (Sp-5,6-DCI-cBIMPS), 8-bromoadenosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cAMPS), and 8-[(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate (Rp-8-CPT-cAMPS). Sigma Chemical Co. provided bovine serum albumin (fraction V), carbocachol, EGTA, HEPES, caffeine, glucagon, human glucagon-like peptide-1 amide fragment 7-36 (GLP-1), 8-bromo-cAMP (8-BrcAMP), 8-bromo-cGMP, and 3-isobutyl-1-methylxanthine (IBMX). Cyclopiazonic acid was from Alexis Corp. (Lausen, Switzerland), and 2-aminoethoxydiphenyl borate (2-APB) was from Aldrich (Gillingham, UK). Fetal calf serum was bought from Invitrogen, and collagenase was from Roche Molecular Biochemicals GmbH. Diazoxide, methoxyverapamil, and forskolin were kindly donated by Schering-Plough Int. (Kenilworth, NJ), Knoll AG (Ludwigshafen, Germany), and Aventis (Stockholm, Sweden) respectively. Membrane polycarbonate filters (25-mm diameter, 25 μm thick with 3-μm pores with a density of 3000/c㎡) were from Osmonics Inc. (Livermore, CA).

**Preparation and Culture of Cells—**Islets of Langerhans were collagenease-isolated from pieces of pancreas from ob/ob mice or Wistar rats. Free cells were prepared by shaking the islets in a Ca\(^{2+}\)-deficient medium. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 30 μg/ml gentamicin and allowed to attach to circular 25-mm cover slips during 1–3 days in culture at 37 °C in a humidified atmosphere of 5% CO\(_2\). The ob/ob mouse islets contain more than 90% β-cells (29), which respond normally to glucose and other regulators of insulin release (30). The selection of β-cells for analysis was based on their size and low nuclear/cytoplasmic ratio compared with the cells secreting glucagon, somatostatin (31, 32), and pancreatic polypeptide (33). Human erythroleukemia 92.1.7 (HEL) cells were obtained from Prof. K. E. O. Åkerman (Uppsala, Sweden) and cultured in suspension in RPMI 1640 medium (34).

**Image Analysis of Cytoplasmic Ca\(^{2+}\)—**In most experiments, loading with the indicator fura-2 was performed during 30-min incubation at 37 °C in a HEPEs-buffered medium (25 mM; pH 7.4) containing 0.5 mg/ml bovine serum albumin, 138 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl\(_2\), 1.28 mM CaCl\(_2\), 20 mM glucose, 250 μM diazoxide, 50 μM methoxyverapamil, and 0.25 μM fura-2/AM. In the experiment shown in Fig. 1A, diazoxide and methoxyverapamil were omitted, and the glucose concentration was 3 mM. Methoxyverapamil was also omitted in the experiments shown in Figs. 1, B–D, and 6A. Testing the effect of rhodamine, 100 μM of this compound was present during loading and throughout the experiment. The cover slips with attached cells were used as exchangeable bottoms of a modified open Sykes-More chamber (35). The chamber profile was defined by a 4-mm wide, 7-mm long oval hole in a 1-mm thick silicone rubber gasket with a 25-mm outer diameter. A thin 25-mm diameter stainless steel plate with an identical central opening pressed the rubber gasket to the coverslip by the threaded Sykes-More chamber mount. Inlet and outlet cannulas fixed to the stainless steel plate allowed laminar flow superfusion. The chamber was placed on the stage of an inverted microscope (Eclipse TE2000U; Nikon, Kanagawa, Japan). The chamber holder and the CFI S Fluor 40 × 1.3 numerical aperture oil immersion objective (Nikon) were maintained at 37 °C by custom-built thermostats. The chamber was superfused at a rate of 0.3 ml/min with the loading medium lacking indicator.

The microscope was equipped with an epifluorescence illuminator (Cairn Research Ltd, Faversham, UK) connected through a 5-mm diameter liquid light guide to an Optoscan monochromator (Cairn Research Ltd) with rapid gratting and slit width adjustment and a 150-watt xenon arc lamp. The monochromator provided excitation light at

![FIG. 1. Depolarization-dependent Ca\(^{2+}\) entry triggers CICR, which is facilitated by cAMP. Mouse (A and B) or rat (C and D) pancreatic β-cells were loaded for 30 min with 0.25 μM fura-2/AM in medium containing 3 (A) or 20 mM glucose (B–D) and 0 (A) or 250 μM diazoxide (D). The cells were then rinsed and superfused with the same medium lacking indicator. As indicated by bars the cells were exposed to 20 mM glucose, 250 μM diazoxide, 17, 30, or 90 mM KCl, 1 μM forskolin, 10 mM GLP-1, and 10 mM glucagon. The induction of Ca\(^{2+}\) spiking in response to 20 mM glucose and 90 mM KCl in A was representative for seven of nine cells (p < 0.01). In B, depolarization with 17 mM KCl alone induced occasional Ca\(^{2+}\) spikes (on average 1.4 per 10 min; p < 0.02; n = 13). Though not shown in B, exposure to forskolin alone induced occasional Ca\(^{2+}\) spiking (on average 1.2 per 10 min; p < 0.02; n = 13). However, the combination of KCl and forskolin induced much more frequent Ca\(^{2+}\) spiking (on average 5.3 per 10 min; p < 0.001; n = 13). In C and D, 5 of 13 cells (p < 0.05) and 7 of 17 cells (p < 0.01), respectively, responded to 30 mM KCl with Ca\(^{2+}\) spiking.](http://www.jbc.org/)
After loading for 30 min, the HEL-cells and β-cell were rinsed in indicator-free medium. The coverslip with β-cells was used as bottom of the chamber described above. In this case, a 0.25-mm thick, 25-mm diameter polyester spacer with a central 4-mm wide and 7-mm oval hole (like that in the silicon rubber gasket; see above) was placed on top of the coverslip. Fura-2-loaded HEL cells were pipetted into the central cavity defined by the hole in the spacer, which was then covered by a 25-mm polycarbonate membrane filter before aligning the hole in the polyester spacer. Fura-2-loaded HEL cells were pipetted into the central cavity defined by the hole in the spacer, which was then covered by a 25-mm polycarbonate membrane filter before aligning the hole in the polyester spacer.

CICR Is Promoted by Glucagon, GLP-1, and other cAMP Agonists—In accordance with earlier data (37, 38), increase of the glucose concentration from 3 to 20 mM induced initial oscillations. Addition of diazoxide, which hyperpolarizes the membrane potential close to the equilibrium potential for K⁺, immediately abolished the slow spikes, indicating that the depolarization-dependent entry of Ca²⁺ spikes in response to Glc (C) and forskolin (D) were reduced by 70% (p < 0.001; n = 16) and 71% (p < 0.001; n = 16) by Rp-8-Br-cAMPS and Rp-8-CPT-cAMPS, respectively.

Statistical Analysis—Only recordings from isolated individual β-cells were included in the analyses. Statistical evaluations of the proportion of cells with a certain response were made with Fishers exact test or χ² test with Yates’ correction using SigmaStat software (SPSS Inc., Chicago, IL). Wilcoxon signed rank test was used to compare the frequency of Ca²⁺ spikes. Statistical significance was set at a p value of < 0.05.
presence of methoxyverapamil to block depolarization-dependent elevation of \( [\text{Ca}^{2+}]_i \) which like most subsequent experiments was performed in the same medium lacking indicator. As indicated by bars, the cells were exposed to 10 nM glucagon, 10 nM GLP-1, 100 \( \mu \text{M} \) IBMX, and 2 or 20 mM caffeine. 20 mM caffeine completely inhibited CICR in response to glucagon (\( A; p < 0.01; n = 9 \)), and IBMX (\( C; p < 0.001; n = 22 \)) and reduced the average frequency of \( [\text{Ca}^{2+}]_i \) spiking in response to GLP-1 by 97% (\( B; p < 0.001; n = 37 \)) and to 2 mM caffeine by 70% (\( D; p < 0.001; n = 19 \)).

However, 8-bromo-cGMP or the more selective protein kinase G activator 8-(4-chlorophenylthio)-cGMP failed to trigger CICR in \( \beta \)-cells subsequently responding to cAMP agonists (not shown).

**PKA Rather Than Epac Mediates the cAMP Effect on CICR**—Occasional \( [\text{Ca}^{2+}]_i \) spikes were observed in 2 of 64 individual islet cells exposed to the Epac-specific activator 8-pCPT-2’-O-Me-cAMP and in 1 of 57 cells exposed to the even more potent 8-pMeOPT-2’-O-Me-cAMP (data not shown). Epac activator-induced \( [\text{Ca}^{2+}]_i \) spikes were sometimes seen also in islet cell clusters. These data indicate that the response represents a different cell type than the dominating \( \beta \)-cells. However, 111 of 174 cells (64%; \( p < 0.001 \)) reacted to the PKA-specific activator Sp-5,6-DCI-cBIMPS with generation of repetitive \( [\text{Ca}^{2+}]_i \) spikes. Fig. 3, A and B, illustrate lack of response to the Epac activators 8-pCPT-2’-O-Me-cAMP and 8-pMeOPT-2’-O-Me-cAMP in cells subsequently reacting to the PKA activator. Further evidence for a PKA mechanism was obtained from the observation that the frequencies of the \( [\text{Ca}^{2+}]_i \) spiking induced by glucagon and forskolin were reduced by about 70% by the competitive PKA antagonists Rp-8-Br-cAMPS (Fig. 3C) and Rp-8-CPT-cAMPS (Fig. 3D).

**cAMP-promoted CICR Is Independent of RyRs and Prevented by IP\(_3\)R Inhibition**—Low concentrations of caffeine have been
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**Fig. 6. CICR is resistant to ryanodine.** Mouse pancreatic \( \beta \)-cells were loaded for 30 min with 0.25 \( \mu \)M fura-2/AM in medium containing 20 mM glucose, 250 \( \mu \)M diazoxide, 100 \( \mu \)M ryanodine (A–C), and 50 \( \mu \)M methoxyverapamil (B and C). The cells were then rinsed and superfused with the same medium lacking indicator. As indicated by bars, the cells were exposed to 90 mM KCl, 50 \( \mu \)M methoxyverapamil, 100 mM ryanodine, 10 mM glucose, 250 \( \mu \)M Sp-5,6-DCI-cBIMPS (Sp-cBIMPS), and 20 mM caffeine. KCl depolarization induced CICR spiking in 15 of 27 cells (A; \( p < 0.001 \)), glucose in 46 of 48 cells (B; \( p < 0.001 \)), and Sp-5,6-DCI-cBIMPS in 35 of 50 cells (C; \( p < 0.001 \)). The presence of caffeine abolished CICR spiking in all 35 Sp-5,6-DCI-cBIMPS-responsive cells (C; \( p < 0.001 \)).

used extensively as phosphodiesterase inhibitor raising cAMP. We now found that 2 mM caffeine mimics other cAMP agonists in inducing CICR (Figs. 2E and 4D). However, high caffeine concentrations interfere with both IP\(_3\) production (39) and IP\(_3\)Rs (40), which explains why 20 mM immediately inhibited CICR promoted by glucose (Fig. 4A), GLP-1 (Fig. 4B), IBMX (Fig. 4C), and 2 mM caffeine (Fig. 4D). In addition, high concentrations of caffeine activated RyRs (41, 42), but 20 mM caffeine never induced an acute mobilization of intracellular Ca\textsuperscript{2+} typically observed in cells with functional RyRs (12, 23).

Because the caffeine data indicate that cAMP-promoted CICR involves the IP\(_3\) signaling pathway, we tested the effect of the membrane-permeable IP\(_3\)R inhibitor 2-APB (43). The inhibitory effect of 2-APB is incomplete (44), and we found that 50 \( \mu \)M prevented Ca\textsuperscript{2+} signaling induced by 10 \( \mu \)M carbachol in 35% of these cells but never the response to 100 \( \mu \)M carbachol (Fig. 5A). However, 50 \( \mu \)M 2-APB inhibited CICR spiking promoted by glucose (Fig. 5B) and forskolin (Fig. 5C).

We also investigated whether pretreatment with a high concentration of ryanodine, which abolishes RyR-mediated CICR in clonal \( \beta \)-cells (12, 23), affects CICR in primary mouse \( \beta \)-cells. Ryanodine neither affected CICR in response to depolarization-dependent elevation of [Ca\textsuperscript{2+}], during exposure to 90 mM KCL (Fig. 6A) nor that induced from basal [Ca\textsuperscript{2+}], levels by glucose (Fig. 6B) or the PKA-specific activator Sp-5,6-DCI-cBIMPS (Fig. 6C). Fig. 6C also shows that addition of 20 mM caffeine inhibits the CICR in response to Sp-5,6-DCI-cBIMPS. The inhibitory effects of caffeine and 2-APB and the lack of effects of ryanodine indicate that the cAMP-promoted CICR is the result of activation of IP\(_3\)Rs rather than RyRs.

**CICR Spikes Trigger Secretion in \( \beta \)-cells—**To record secretory events from the mouse \( \beta \)-cells, we used HEL cells as reporter cells. The HEL cells are not electrically excitatory but show robust [Ca\textsuperscript{2+}], responses to many neurotransmitters (34), including ATP (45), which is released together with insulin from the \( \beta \)-cell secretory granules (46). Fig. 7 shows recordings of [Ca\textsuperscript{2+}], in two \( \beta \)-cells and surrounding HEL cells. The cells were stimulated with 11 mM glucose, and [Ca\textsuperscript{2+}], was kept low in the beginning of the experiment by the presence of diazoxide. Depolarization with 90 mM KCl resulted in elevation of [Ca\textsuperscript{2+}], in the \( \beta \)-cells with superimposed spikes caused by CICR. The adjacent HEL cells did not respond to the depolarization, but some HEL cells reacted with [Ca\textsuperscript{2+}], spikes following spikes in the closest \( \beta \)-cell. In the \( \beta \)-cell labeled \( \beta \)-1, the first [Ca\textsuperscript{2+}], spike was followed within one acquisition cycle (2 s) by spikes in the cells labeled HEL-1 and HEL-2 near \( \beta \)-1, but these HEL cells did not respond to two subsequent spikes in \( \beta \)-1. In the \( \beta \)-cell labeled \( \beta \)-2, the second and the last [Ca\textsuperscript{2+}], spikes were followed by spikes within 2 s in the HEL-3 cell, which is closest. Moreover, the HEL-4 cells located about one cell diameter away responded to the last spike in the \( \beta \)-2 within 4 s. Of 16 Ca\textsuperscript{2+} spikes in eight HEL cells, they always occurred shortly after spikes in five nearby \( \beta \)-cells.

**DISCUSSION**

The hormones GLP-1 and glucagon potently amplify nutrient-stimulated insulin secretion by raising cAMP, which interacts with a plethora of signal transduction processes, including ion channel activity, intracellular Ca\textsuperscript{2+} handling, and exocytosis of the insulin-containing granules (47). As shown here and demonstrated elsewhere (24, 48), elevation of cAMP promotes [Ca\textsuperscript{2+}], spiking superimposed on depolarization-dependent Ca\textsuperscript{2+} entry in glucose-stimulated \( \beta \)-cells, an effect originally attributed to mobilization of intracellular Ca\textsuperscript{2+} after sensitization of IP\(_3\)Rs (24) by a PKA mechanism (25). However, when this action of cAMP was first conceptually associated with CICR, it was instead assumed to represent PKA-dependent phosphorylation of RyRs (9, 10). Maintaining the idea that RyRs are involved, the role of cAMP was later reconsidered, claiming that the receptor activation is caused by a PKA-independent Epac mechanism (20, 22, 27).

Although opinions differ with regard to the type of receptors, there seems to be general agreement that cAMP promotes CICR by receptor sensitization. Such a mechanism implies that cAMP not only promotes CICR, it was instead assumed to represent PKA-dependent phosphorylation of RyRs (9, 10). Maintaining the idea that RyRs are involved, the role of cAMP was later reconsidered, claiming that the receptor activation is caused by a PKA-independent Epac mechanism (20, 22, 27).

Eliminating interference from depolarization-dependent Ca\textsuperscript{2+} influx, we first studied whether cAMP acts via PKA or Epac. Our data unequivocally favored PKA. Only 2–3% of the islet cells reacted with one or two Ca\textsuperscript{2+} spikes when exposed to two potent Epac activators, suggesting that this response originated from cells other than the dominating \( \beta \)-cells. However, a specific activator of PKA induced repetitive CICR spikes in 64% of the islet cells. Moreover, two competitive PKA antagonists inhibited CICR spiking in response to cAMP elevation by about...
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Fig. 7. CICR triggers exocytosis. HEL cells in suspension were loaded with 0.5 \(\mu\)M fura-2/AM during incubation for 30 min. Mouse pancreatic \(\beta\)-cells attached to a coverslip were loaded for 30 min with 1 \(\mu\)M fluo-4/AM in medium containing 11 mM glucose and 250 \(\mu\)M diazoxide. After rinsing in the latter medium lacking indicator, HEL cells were added to the chamber with \(\beta\)-cells and covered by a permeable polycarbonate filter. The cells were then superfused with the same medium lacking indicator. As indicated by the bar, the cells were exposed to 90 mM KCl. The drawing in A illustrates the relative positions of the studied \(\beta\)-cells and HEL cells, and the traces in B show the measurements of [Ca\(^{2+}\)]\(_i\) in each cell. The [Ca\(^{2+}\)]\(_i\) data in \(\beta\)-cells are presented as \(\Delta F/F_0\), which corresponds to the average basal fluorescence before the addition of KCl and \(\Delta F\) to the deviation from \(F_0\). The dashed vertical lines illustrate the delay between spikes in \(\beta\)-cells and HEL cells. Of 32 Ca\(^{2+}\) spikes in five \(\beta\)-cells, 16 were followed by spikes in eight nearby responsive HEL cells (\(p < 0.001\)).

70%. Although a stimulatory effect of CO on Ca\(^{2+}\) spiking in \(\beta\)-cells exposed to glucon has been taken to indicate that cGMP promotes intracellular mobilization of Ca\(^{2+}\) (49), we found no effect of protein kinase G agonists. Protein kinase G activation alone is consequently insufficient for promoting CICR under the present conditions.

We proceeded to study the type of receptor involved in CICR after elevation of cAMP. Caffeine was one tool in this exploration. The classic effect of caffeine on \(\beta\)-cells is phosphodiesterase inhibition with elevation of cAMP (50). Therefore, it is not surprising that a low concentration of caffeine mimicked the effect of other cAMP agonists in promoting CICR. However, caffeine also sensitizes RyRs to Ca\(^{2+}\) (42) and high concentrations even activate RyRs independent of Ca\(^{2+}\) (41). Our observation that 20 mM caffeine failed to mobilize intracellular Ca\(^{2+}\) in \(\beta\)-cells exposed to cAMP agonists argues against the involvement of RyRs in CICR. This conclusion was further supported by the lack of effect of ryanodine pretreatment on CICR induced by depolarization-dependent elevation of [Ca\(^{2+}\)]\(_i\), by elevation of cAMP, or by direct PKA activation. We have previously shown that ryanodine pretreatment abolishes caffeine-induced CICR in clonal \(\beta\)-cells, which express functional RyRs (23). As a result, the present data do not support the idea that elevation of cAMP uncovers CICR by an action on RyRs (9, 10).

Apart from the above-mentioned actions, caffeine interferes with IP\(_3\) signaling. High concentrations thus inhibit agonist-induced formation of IP\(_3\) (39) as well as its action on the IP\(_3\)-Rs (40), an effect observed also in pancreatic \(\beta\)-cells (51). Because of the opposite actions on RyRs and IP\(_3\)-Rs, high concentrations of caffeine have been used to discriminate between them (52). Our observation that 20 mM caffeine inhibits cAMP/PKA-promoted CICR is therefore consistent with an IP\(_3\)-R-mediated effect. This idea was further tested with the cell-permeable IP\(_3\)-R antagonist 2-APB (43). Despite its limited potency (44), 2-APB strongly inhibited Ca\(^{2+}\) spiking promoted by glucagon and forskolin, providing additional arguments for the involvement of IP\(_3\)-Rs in CICR promoted by cAMP.

Glucose-stimulated insulin secretion depends on influx of Ca\(^{2+}\) through voltage-dependent L-type channels. Evidence indicates a close association between these channels and the secretory granules (53). Thanks to this arrangement, the [Ca\(^{2+}\)]\(_i\) level triggering exocytosis of the granules reaches 5–10-fold higher concentrations than in the remainder of the cytoplasm. The present experiments show that [Ca\(^{2+}\)]\(_i\) spikes caused by CICR in depolarized \(\beta\)-cells trigger a [Ca\(^{2+}\)]\(_i\) response in neighboring reporter cells. Because this response was always delayed by at least one acquisition cycle (2 s), it probably represents release of an active messenger from the \(\beta\)-cells. This factor may be ATP, which is co-secreted with insulin (46) and is known to induce a purinergic Ca\(^{2+}\) response in HEL cells (45). Our data consequently show that CICR is an amplifier of exocytosis in response to depolarization-dependent influx of Ca\(^{2+}\), perhaps indicating that CICR is acting locally to further elevate the high [Ca\(^{2+}\)]\(_i\) levels at the site of exocytosis. The failure of some [Ca\(^{2+}\)]\(_i\) spikes to elicit a response in the HEL cells may indicate that secretion does not always occur or that the content of active factor(s) varies between secretory granules. If secretion occurs from a \(\beta\)-cell site not facing the reporter cell, it is also possible that dilution in the medium prevents a response. Indeed, the HEL cells are apparently not sufficiently sensitive to detect secretion from the \(\beta\)-cells in the absence of CICR, although [Ca\(^{2+}\)]\(_i\), is elevated by depolarization alone.

A recent study of mouse \(\beta\)-cells indicates the presence of an atypical CICR mechanism involving neither IP\(_3\)-Rs nor RyRs (12). This phenomenon is rather sluggish and distinctly different from the explosive Ca\(^{2+}\) spiking characterizing the primarily studied CICR. Moreover, knockout of the low-affinity Ca\(^{2+}\)-transporting sarco/endoplasmic reticulum ATPase 3 (SERCA-3) abolishes the atypical CICR (12) but does not affect IP\(_3\)-mediated Ca\(^{2+}\)-release, which depends on the high-affinity SERCA-2 (54). Because SERCA-3 is activated only when [Ca\(^{2+}\)]\(_i\) is elevated above basal levels (54), the presently used conditions with diazoxide and methoxyverapamil would prevent filling of the Ca\(^{2+}\) pool from which the atypical CICR occurs.

In most cases, controversies regarding the type of receptor underlying CICR in pancreatic \(\beta\)-cells can be explained by the involvement of different mechanisms in clonal and primary \(\beta\)-cells (23). Nearly all published data on CICR in primary \(\beta\)-cells are consistent with a PKA-mediated effect on IP\(_3\)-Rs, even when the authors favor RyRs. The observations that the
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Epac activator 8-pCPT-2'-O-Me-cAMP raises [Ca\(^{2+}\)], and stimulates exocytosis in human \(\beta\)-cells were attributed to activation of CICR via RyRs (22). It is unfortunate that the close proximity periods (10 s) to Epac activator preclude discrimination between activation of Ca\(^{2+}\) influx and release from a limited intracellular pool. Such discrimination is also prevented by exposure to 5.6 mM glucose, which depolarizes the \(\beta\)-cell to the threshold for opening of L-type Ca\(^{2+}\) channels and stimulation of insulin release. Under such conditions, any minor depolarization triggers Ca\(^{2+}\) influx. Indeed, by inhibiting the K\(_\text{ATP}\) channels, cAMP depolarizes the \(\beta\)-cell and induces electrical activity even at subthreshold concentrations of glucose (55). Moreover, CAMP amplifies Ca\(^{2+}\) influx into the \(\beta\)-cells by a direct effect on the L-type channels (56). It remains to establish whether Epac is involved in these cAMP effects on the KATP channel. Although ryanodine was reported to diminish the Ca\(^{2+}\)-elevating action of 8-pCPT-2'-Me-cAMP on the \(\beta\)-cells (57), 8-pCPT-2'-Me-cAMP on the KATP channels were attributed to activation of CICR even after elevation of cAMP. GLP-1 shows a promising role than CICR (14), we find no evidence for their involvement. Moreover, cAMP amplifies Ca\(^{2+}\) influx into the \(\beta\)-cells by a direct effect on the L-type channels (56). It remains to establish whether Epac is involved in these cAMP effects on the KATP channel and CICR (14).

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