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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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Running title: Ca^{2+}-Induced Ca^{2+} Release in Primary Pancreatic β-cells

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Hormones, like 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, since 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 (PKA) in cells unresponsive to activators of cAMP-regulated guanine nucleotide exchange factor. Ryanodine pretreatment, which abolishes CICR mediated by ryanodine receptors (RyRs), did not prevent CICR. Moreover, a high concentration of caffeine, known to activate RyRs independent 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). Accordingly, the cell permeable IP_3R 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 erythroleukemia cells, used to report secretory events in the β-cells. The results indicate that PKA-mediated promotion of CICR via IP_3Rs is part of the mechanism by which cAMP amplifies insulin release.

Keywords: Ca^{2+}-induced Ca^{2+} release, IP_3 receptors, Ryanodine receptors, Cyclic AMP, Protein kinase A, Insulin secretion, Endoplasmic reticulum, Calcium

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 due to closure of ATP/ADP-sensitive K^+ (K_{ATP})[1] 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 has also 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 due to activation of phospholipase C, catalysing 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 the classical 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 sarcoplasmic reticulum (15). In heart cells CICR is due to activation of RyRs. However, in many other types of cells IP_3Rs are equally competent in mediating CICR, since 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).

Like 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 tumour-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 due to 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 exchange factor (Epac) has been suggested (20,22,27). Since important evidence for this concept was obtained with clonal β-cells we have now studied the mechanisms by which cAMP promotes CICR in primary mouse and rat β-cells. We show that cAMP-facilitated CICR is due to PKA-dependent activation of IP_3Rs. 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 acetoxymethyl ester (fura-2/AM), fluo-4 acetoxymethyl ester (fluo-4/AM) and ryanodine were from Molecular Probes Inc. (Eugene, OR). Biolog Life Science Institute (Bremen, Germany) was the source of 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP), 8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP), 8-(4-methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclicmonophosphate (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-DCl-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. (St. Louis, MO) provided bovine serum albumin (fraction V), carbachol, EGTA,

HEPES, caffeine, porcine glucagon, human glucagon-like peptide-1 amide fragment 7-36 (GLP-1), 8-bromoadenosine-3',5'- cyclic monophosphate (8-Br-cAMP), 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP) and 3-isobutyl-1-methylxanthine (IBMX). Cyclopiazonic acid (CPA) was from Alexis Corp. (Lausen, Switzerland) and 2-aminoethoxydiphenyl borate (2-APB) from Aldrich (Gillingham, UK). Fetal calf serum was bought from Gibco Ltd. (Paisley, Scotland) and collagenase was from Boehringer Mannheim GmbH (Mannheim, Germany). Diazoxide, methoxyverapamil and forskolin were kindly donated by Schering-Plough Int. (Kenilworth, NJ), Knoll AG (Ludwigshafen, Germany) and Hoechst Marion Roussels (Stockholm, Sweden) respectively. Membrane polycarbonate filters (25 mm diameter, 25 μm thick with 3 μm pores with a density of 3000/cm^2) were from Osmonics Inc. (Livemore, CA).

Preparation and culture of cells--Islets of Langerhans were collagenase-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 (28). The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and 30 mg/ml gentamicin, and allowed to attach to circular 25 mm cover slips during 1-3 days 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 large 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 Professor 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 of cells 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. 1B-D and 6A. Testing the effect of ryanodine, 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 and 7 mm long oval hole in a 1 mm thick silicon rubber gasket with 25 mm outer diameter. A thin 25 mm diameter stainless steel plate with an identical central opening pressed the rubber gasket to the cover slip by the threaded Sykes-More chamber mount. Inlet and outlet cannulas fixed to the stainless steel plate allowed close to 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 40x 1.3 NA oil immersion objective (Nikon) were maintained at 37°C by custom-built thermostats. The chamber was superfused at a rate of 0.3 ml/minute 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 grating and slit width adjustment and a 150W xenon arc lamp. The monochromator provided excitation light at 340 nm (1.7 nm half bandwidth) and 380 nm (1.4 nm half bandwidth). Emission was measured at 510 nm (40 nm half bandwidth) using a 400 nm dichroic beam splitter and a cooled OrcaER-1394 firewire digital CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) equipped with a C8600-2 image intensifier (Hamamatsu Photonics). The Metafluor software (Universal Imaging Corp. Downingtown, Pa) controlled the monochromator and the CCD camera,

acquiring pairs of 340 and 380 nm images every 2 sec with integration for 60-80 msec at each wavelength and <1 msec for changing wavelength and slits. To minimize bleaching and photo damage, the monochromator slits were closed until the start of the next acquisition cycle. Ratio (*R*) images were calculated after subtraction of background images. $[Ca^{2+}]_i$ values were obtained according to Grynkiewicz et al. (36) using the equation

$$[Ca^{2+}]_i = K_D^{Ca^{2+}} \cdot \frac{F_0}{F_S} \cdot \frac{(R - R_{\min})}{(R_{\max} - R)}$$

$K_D^{Ca^{2+}}$ is 224 nM. F_0 and R_{min} are the fura-2 fluorescence at 380 nm and the 340/380 nm fluorescence excitation ratio, respectively, in an "intracellular" K^+-rich medium lacking Ca^{2+}. F_S and R_{max} are the corresponding data obtained with a saturating concentration of Ca^{2+}.

Detection of β-cell secretion--To record secretory events from the mouse β-cells we used HEL cells as reporter cells. The suspension-cultured HEL cells were spun down and loaded with 0.5 μM fura-2 as previously described (34). β-Cells attached to cover slips were loaded with the single wavelength Ca^{2+} indicator fluo-4 by incubation in medium (similar as used for β-cells above) containing 11 mM glucose, 250 μM diazoxide, 1 μM fluo-4/AM but lacking methoxyverapamil. After loading for 30 min the HEL-cells and β-cell were rinsed in indicator-free medium. The cover slip 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 in the silicon rubber gasket; see above) was placed on top of the cover slip. 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 silicon rubber gasket and the stainless steel plate (see above) with the hole in the polyester spacer. β-Cells attached to the cover slip and

free-floating HEL cells under the permeable polycarbonate filter could then be superfused with minimal cell movements. The principal arrangement has previously been described (34) but the currently used chamber had a different geometry. Fluorescence was measured essentially as described above with emission at >510 nm (long-pass filter) using a 495 nm dichroic beam splitter. Fura-2 fluorescence from the HEL cells was excited at 340 and 380 nm and fluo-4 fluorescence from the β-cells at 470 nm. Images were obtained every 2 sec with integration for 100 msec at each wavelength and <1 msec for changing wavelength and slits. Due to the stronger fluorescence from fluo-4 the image intensifier gain was reduced during exciation at 470 nm to balance the signals. $[Ca^{2+}]_i$ in the HEL cells was calculated as described above. The fluo-4 fluorescence intensity indicating $[Ca^{2+}]_i$ variations in the β-cells was expressed as the ratio between deviation from the basal fluorescence and the basal fluorescence ($\Delta F/F_0$).

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 χ^2 test with Yates' correction using SigmaStat software (SPSS Inc., Chicago, IL). Wilcoxon signed rank test was used to compare the frequency of Ca^{2+} spikes. Statistical significance was set at a *P* value of < 0.05.

RESULTS

CICR is promoted by glucagon, GLP-1 and other cAMP agonists --In accordance with earlier data (37,38) rise of the glucose concentration from 3 to 20 mM induced initial lowering of $[Ca^{2+}]_i$ in β-cells followed by slow large amplitude oscillations of $[Ca^{2+}]_i$ (Fig. 1A). The use of a low indicator concentrations facilitated the detection of rapid $[Ca^{2+}]_i$ spikes, which were superimposed on top of the large amplitude oscillations. Addition of diazoxide, which hyperpolarizes the β-cells by opening K_{ATP} channels, immediately abolished the slow

$[Ca^{2+}]_i$ oscillations as well as the spikes. In the presence of diazoxide the membrane potential is close to the equilibrium potential for K^+. Under these conditions depolarization with 90 mM KCl induced a rapid rise of $[Ca^{2+}]_i$ with superimposed $[Ca^{2+}]_i$ spikes, indicating that the depolarization-dependent influx of Ca^{2+} triggers CICR. Most subsequent experiments were performed in the presence of 20 mM glucose to stimulate Ca^{2+} sequestration in the ER (2-4) and diazoxide to keep the membrane potential close to the equilibrium potential for K^+. Fig. 1B illustrates that the modest elevation of $[Ca^{2+}]_i$ obtained with 17 mM KCl only triggered occasional CICR spikes. After return to the physiological K^+ concentration addition of a low concentration of the adenylyl cyclase activator forskolin evoked occasional CICR spikes (not shown). However, combining 17 mM KCl with forskolin increased the frequency of CICR spiking 4-fold. The ability of cAMP to uncover CICR in response to depolarization-dependent rise of $[Ca^{2+}]_i$ was not restricted to mouse β-cells. Figs. 1C and D illustrate $[Ca^{2+}]_i$ spiking in rat β-cells depolarized with 30 mM KCl in the presence of the adenylyl cyclase-activating hormones GLP-1 and glucagon respectively. However, the mouse β-cells were more sensitive and the same concentrations of glucagon and GLP-1 caused repetitive CICR spiking even in the absence of depolarization-dependent elevation of $[Ca^{2+}]_i$. This is illustrated in Fig. 2, which like most subsequent experiments was performed in the presence of methoxyverapamil to block depolarization-dependent Ca^{2+} entry and keep $[Ca^{2+}]_i$ at basal levels. Apart from the physiological activation of adenylyl cyclase with glucagon (Fig. 2A) and GLP-1 (Fig. 2B) CICR spiking from the baseline was also obtained after direct activation of adenylyl cyclase with forskolin (Fig. 2C), by inhibition of cAMP degradation with the phosphodiesterase inhibitors IBMX (Fig. 2D) or caffeine (Fig. 2E) and by cell membrane-permeable 8-Br-cAMP (Fig. 2F). However, 8-Br-cGMP or the more selective protein kinase G (PKG) activator 8-pCPT-cGMP failed to trigger CICR in β-cells subsequently responding to cAMP agonists (not shown).

PKA rather than Epac mediates the cAMP effect on CICR--Occasional Ca^{2+} 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 (not shown). Epac activator-induced Ca^{2+} spikes were sometimes seen also in islet cell clusters. These data indicate that the response represents a different cell type than the dominating β-cells. However, 111 of 174 cells (64%; $P<0.001$) reacted to the PKA-specific activator Sp-5,6-DCl-cBIMPS with generation of repetitive $[Ca^{2+}]_i$ spikes. Figs. 3A 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 Ca^{2+} spiking induced by GLP-1 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_3R inhibition--Low concentrations of caffeine have been used extensively as phosphodiesterase inhibitor raising cAMP. We now found that 2 mM caffeine mimics other cAMP agonists in inducing CICR (Figs. 2E, 4D). However, high caffeine concentrations interfere both with IP_3 production (39) and IP_3Rs (40) explaining why 20 mM immediately inhibited CICR promoted by glucagon (Fig. 4A), GLP-1 (Fig. 4B), IBMX (Fig. 4C) and 2 mM caffeine (Fig. 4D). In addition high concentrations of caffeine activate RyRs (41,42) but 20 mM caffeine never induced an acute mobilization of intracellular Ca^{2+} typically observed in cells with functional RyRs (12,23).

Since the caffeine data indicate that cAMP-promoted CICR involves the IP_3 signaling pathway we tested the effect of the membrane-permeable IP_3R inhibitor 2-APB (43). The inhibitory effect of 2-APB is incomplete (44) and we found that 50 μM prevented Ca^{2+} signaling induced by 10 μM carbachol in 35 % of the cells but never the response to 100 μM

carbachol (Fig. 5A). However, 50 μM 2-APB inhibited CICR spiking promoted by glucagon (Fig. 5B) and forskolin (Fig. 5C).

We also investigated whether pretreatment with a high concentration of ryanodine, which abolishes RyR-mediated CICR in clonal β-cells (12,23), affects CICR in primary mouse β-cells. Ryanodine neither affected CICR in response to depolarization-dependent elevation of $[Ca^{2+}]_i$ during exposure to 90 mM KCl (Fig. 6A), nor that induced from basal $[Ca^{2+}]_i$ levels by glucagon (Fig. 6B) or the PKA-specific activator Sp-5,6-DCl-cBIMPS (Fig. 6C). Fig. 6C also shows that addition of 20 mM caffeine inhibits the CICR in response to Sp-5,6-DCl-cBIMPS. The inhibitory effects of caffeine and 2-APB and the lack of effects of ryanodine indicate that the cAMP-promoted CICR is due to activation of IP_3Rs rather than RyRs.

CICR spikes trigger secretion in β-cells--To record secretory event from the mouse β-cells we used HEL cells as reporter cells. The HEL cells are not electrically excitable but show robust $[Ca^{2+}]_i$ responses to many neurotransmitters (34) including ATP (45), which is released together with insulin from the β-cell secretory granules (46). Fig. 7 shows recordings of $[Ca^{2+}]_i$ in two β-cells and surrounding HEL cells. The cells were stimulated with 11 mM glucose, and $[Ca^{2+}]_i$ was kept low in the beginning of the experiment by the presence of diazoxide. Depolarization with 90 mM KCl resulted in elevation of $[Ca^{2+}]_i$ in the β-cells with superimposed spikes due to CICR. The adjacent HEL cells did not respond to the depolarization but some HEL cells reacted with $[Ca^{2+}]_i$ spikes following spikes in the closest β-cell. In the β-cell labeled as β-1 the first $[Ca^{2+}]_i$ spike was followed within one acquisition cycle (2 sec) by spikes in the cells labeled as HEL-1 and HEL-2 close to β-1, but these HEL cells did not respond to two subsequent spikes in β-1. In the β-cell labeled as β-2 the second and the last $[Ca^{2+}]_i$ spikes were followed by spikes within 2 sec 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 β-2 within 4 sec. Analyzing 16 Ca^{2+} spikes in 8 HEL cells, they always occurred shortly after spikes in 5 closely located β-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^{2+} handling and exocytosis of the insulin-containing granules (47). As shown here and demonstrated elsewhere (24,48) elevation of cAMP promotes $[Ca^{2+}]_i$ spiking superimposed on depolarization-dependent Ca^{2+} entry in glucose-stimulated β-cells, an effect originally attributed to mobilization of intracellular Ca^{2+} after sensitization of IP_3Rs (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 due to a PKA-independent Epac mechanism (20,22,27).

Although there are different opinions 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 enables CICR to be triggered at lower concentrations of Ca^{2+}. The present data indicate that cAMP not only promotes CICR in response to depolarization-dependent elevation of $[Ca^{2+}]_i$ but sensitizes the underlying mechanisms sufficiently for CICR to occur from basal levels of $[Ca^{2+}]_i$ in β-cells exposed to hyperpolarizing diazoxide and to the voltage-dependent Ca^{2+} channel blocker methoxyverapamil. This is a favorable situation, since CICR can be studied under conditions allowing discrimination between depolarization-dependent influx and intracellular release of Ca^{2+}, which was not possible with some previously used protocols.

Eliminating interference from depolarization-dependent Ca^{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^{2+} spikes when exposed to two potent Epac activators suggesting that this response originated from other cells than the dominating β-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 70%. Although a stimulatory effect of CO on Ca^{2+} spiking in β-cells exposed to glucagon has been taken to indicate that cGMP promotes intracellular mobilization of Ca^{2+} (49), we found no effect of PKG agonists. PKG 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 classical effect of caffeine on β-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 β-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 β-cells, which express functional RyRs (23). Consequently, 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_3Rs (40), an effect observed also in pancreatic β-cells (51). Due to the opposite actions on RyRs and IP_3Rs, 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_3R-mediated effect. This idea was further tested with the cell permeable IP_3R antagonist 2-APB (43). Despite of its limited potency (44) 2-APB strongly inhibited Ca^{2+} spiking promoted by glucagon and forskolin, providing additional arguments for the involvement of IP_3Rs in CICR promoted by cAMP.

Glucose-stimulated insulin secretion depends on influx of Ca^{2+} through voltage-dependent L-type channels. There is evidence indicating 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 due to CICR in depolarized β-cells trigger a $[Ca^{2+}]_i$ response in neighboring reporter cells. Since this response was always delayed by at least one acquisition cycle (2 sec), it probably represents release of an active messenger from the β-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 β-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 β-cells in the absence of CICR, although $[Ca^{2+}]_i$ is elevated due to depolarization alone.

A recent study of mouse β-cells indicates the presence of an atypical CICR mechanism neither involving IP_3Rs nor RyRs (12). This phenomenon is rather sluggish and distinctly different from the explosive Ca^{2+} spiking characterizing the presently studied CICR. Moreover knockout of the low affinity Ca^{2+}-transporting sarco(endo)plasmic reticululum 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). Since SERCA-3 is only activated 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 β-cells can be explained by the involvement of different mechanisms in clonal and primary β-cells (23). Nearly all published data on CICR in primary β-cells are consistent with a PKA-mediated effect on IP_3Rs even when the authors favor RyRs. The observations that the Epac activator 8-pCPT-2'-O-Me-cAMP raises $[Ca^{2+}]_i$ and stimulates exocytosis in human β-cells were attributed to activation of CICR via RyRs (22). Unfortunately, the short exposure periods (10 sec) to Epac activator precludes 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 β-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_{ATP} channels, cAMP depolarizes the β-cell and induces electrical activity even at subthreshold concentrations of glucose (55). Moreover, cAMP amplifies Ca^{2+} influx into the β-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 K_{ATP} and Ca^{2+} channels. Although ryanodine was reported to diminish the Ca^{2+}-elevating action of 8-pCPT-2'-O-Me-cAMP on the human β-cells (22), ryanodine has no

effect on CICR elicited by Ca^{2+} leakage from the ER in mouse and human β-cells (23). A stimulatory effect of Epac activation on insulin release does not require participation of CICR since there is evidence that Epac is involved in distal steps of exocytosis explaining how cAMP amplifies Ca^{2+}-triggered secretion (57,58).

We have previously demonstrated that typical CICR in primary pancreatic β-cells from mice rats and humans is due to activation of IP_3Rs. The present data show that this is the case also for cAMP-promoted CICR and that the effect is mediated by PKA. Consistent with a recent suggestion that RyRs in β-cells may not depend on cAMP and have a different role than CICR (14), we find no evidence for their involvement in CICR even after elevation of cAMP. GLP-1 shows promising results in the treatment of type 2 diabetes (59). The present data indicate that part of this effect may be due to activation of CICR, which amplifies insulin release.

Legends to Figures

FIG. 1. Depolarization-dependent Ca^{2+} entry triggers CICR, which is facilitated by cAMP. Mouse (A, B) or rat (C, 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), 0 (A) or 250 μM diazoxide (B-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 nM GLP-1 and 10 nM glucagon. The induction of Ca^{2+} spiking in response to 20 mM glucose and 90 mM KCl in panel A was representative for 7 of 9 cells ($P<0.01$). In panel B depolarization with 17 mM KCl alone induced occasional Ca^{2+} spikes (on average 1.4 per 10 min; $P<0.02$; n=13). Although not shown in panel B also 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 panels 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.

FIG. 2. cAMP promotes CICR even in the absence of depolarization-dependent entry. Mouse pancreatic β-cells were loaded for 30 min with 0.25 μM fura-2/AM in medium containing 20 mM glucose, 250 μM diazoxide and 50 μM methoxyverapamil. The cells were then rinsed and superfused with the same medium lacking indicator. As indicated by bars the cells were exposed to 10 nM glucagon (A), 10 nM GLP-1 (B), 5 μM forskolin (C), 100 μM IBMX (D), 2 mM caffeine (E) or 1 mM 8-Br-cAMP (F). The results are representative for 229 of 296 (A), 143 of 174 (B), 220 of 272 (C), 43 of 68 (D), 19 of 37 (E) and 72 of 132 (F) cells. $P<0.001$ in all cases.

FIG. 3. The cAMP effect on CICR is mediated by PKA. Mouse pancreatic β-cells were loaded for 30 min with 0.25 μM fura-2/AM in medium containing 20 mM glucose, 250 μM diazoxide and 50 μM methoxyverapamil. The cells were then rinsed and superfused with the same medium lacking indicator. As indicated by bars the cells were exposed to 100 μM 8-pCPT-2'-O-Me-cAMP (CPT-Me-cAMP), 100 μM 8-pMeOPT-2'-O-Me-cAMP (OPT-Me-cAMP), 100 μM Sp-5,6-DCl-cBIMPS (Sp-BIMPS), 1 nM glucagon, 5 μM forskolin, 500 μM Rp-8-Br-cAMPS (Rp-Br-cAMPS) and 100 μM Rp-8-CPT-cAMPS (Rp-CPT-cAMPS). The lack of responses to 8-pCPT-2'-O-Me-cAMP (A) and 8-pMeOPT-2'-O-Me-cAMP (B) were representative for 8 of 9 and 30 of 31 cells, respectively, subsequently responding to Sp-5,6-DCl-cBIMPS. The average frequencies of $[Ca^{2+}]_i$ spikes in response to glucagon (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.

FIG. 4. cAMP-promoted CICR is blocked by a high concentration of caffeine. Mouse pancreatic β-cells were loaded for 30 min with 0.25 μM fura-2/AM in medium containing 20 mM glucose, 250 μM diazoxide and 50 μM methoxyverapamil. The cells were then rinsed and superfused with the same medium lacking indicator. As indicated by bars the cells were exposed to 10 nM glucagon, 10 nM GLP-1, 100 μM 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 Ca^{2+} 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).

FIG. 5. IP_3-induced Ca^{2+} release and cAMP-promoted CICR are blocked by 2-APB. Mouse pancreatic β-cells were loaded for 30 min with 0.25 μM fura-2/AM in medium

containing 20 mM glucose, 250 μM diazoxide and 50 μM methoxyverapamil. The cells were then rinsed and superfused with the same medium lacking indicator. As indicated by bars the cells were exposed to 10 or 100 μM carbachol, 10 nM glucagon, 5 μM forskolin and 50 μM 2-APB. 2-APB prevented the $[Ca^{2+}]_i$ response to 10 μM cabachol in 14 of 40 cells ($P<0.001$) but not the response to 100 μM cabachol (A). Although 2-APB seems to completely block CICR spiking in response to glucagon (B) and forskolin (C), spiking remained in some cells and the average frequencies were reduced by 73% ($P<0.001$; n=24) and 86% ($P<0.001$; n=31), respectively.

FIG. 6. CICR is resistant to ryanodine.

Mouse pancreatic β-cells were loaded for 30 min with 0.25 μM fura-2/AM in medium containing 20 mM glucose, 250 μM diazoxide, 100 μM ryanodine (A-C) and 50 μM methoxyverapamil (B, 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 μM methoxyverapamil, 100 μM ryanodine, 10 nM glucagon, 250 μM Sp-5,6-DCl-cBIMPS (Sp-BIMPS) and 20 mM caffeine. KCl depolarization induced CICR spiking in 15 of 27 cells (A; $P<0.001$), glucagon in 46 of 48 cells (B; $P<0.001$) and Sp-5,6-DCl-cBIMPS in 35 of 50 cells (C; $P<0.001$). The presence of caffeine abolished CICR spiking in all 35 Sp-5,6-DCl-cBIMPS-responsive cells (C; $P<0.001$).

FIG. 7. CICR triggers exocytosis. HEL cells in suspension were loaded with 0.5 μM fura-2/AM during incubation for 30 min. Mouse pancreatic β-cells attached to a cover slip were loaded for 30 min with 1 μM fluo-4/AM in medium containing 11 mM glucose and 250 μM diazoxide. After rinsing in the latter medium lacking indicator HEL cells were added to the chamber with β-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 β-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 β-cells are presented as $\Delta F/F_0$ with F_0 corresponding to the average basal fluorescence before the addition of KCl and ΔF to the deviation from F_0. The dashed vertical lines illustrate the delay between spikes in β-cells and HEL cells. Analyzing 32 Ca^{2+} spikes in 5 β-cells 16 were followed by spikes in 8 closely located responsive HEL cells ($P<0.001$).

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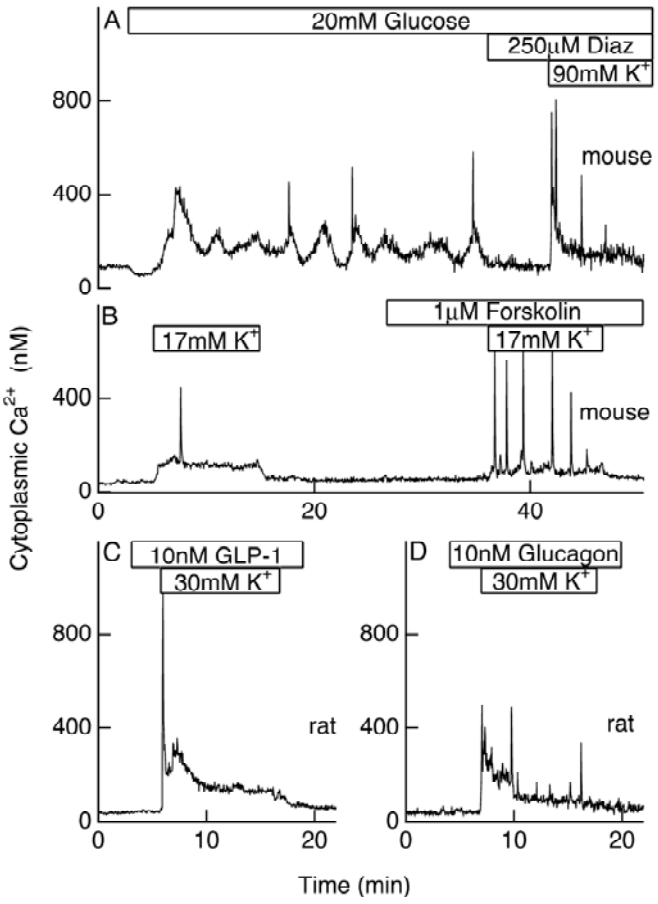
A
20mM Glucose
250μM Diaz
90mM K+
800
400
0
mouse
B
17mM K+
1μM Forskolin
17mM K+
mouse
0
20
40
Cytoplasmic Ca2+ (nM)
C
10nM GLP-1
30mM K+
rat
D
10nM Glucagon
30mM K+
rat
0
10
20
Time (min)

FIG. 1

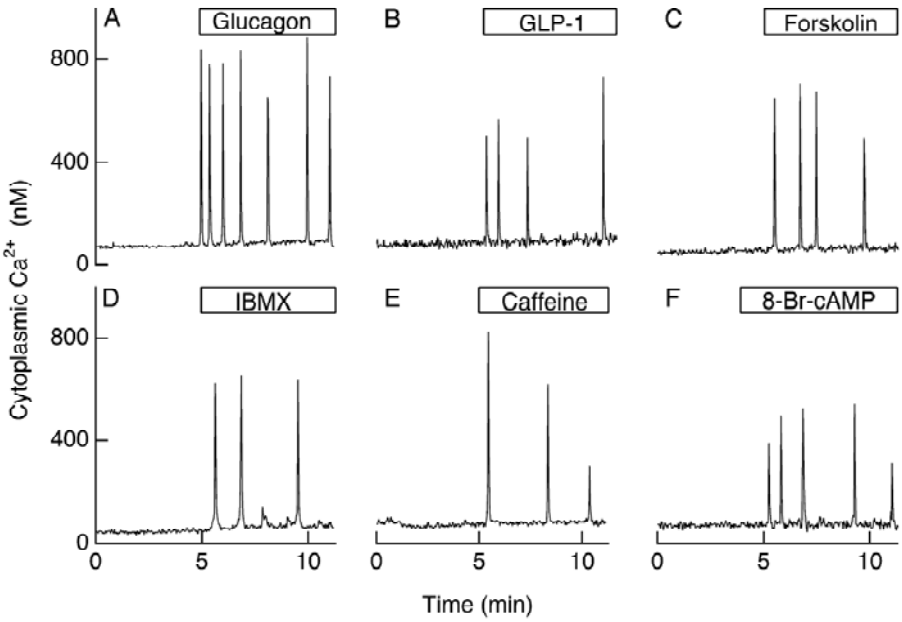
A
Glucagon
B
GLP-1
C
Forskolin
D
IBMX
E
Caffeine
F
8-Br-cAMP
800
400
0
Cytoplasmic Ca^{2+} (nM)
0
5
10
Time (min)

FIG. 2

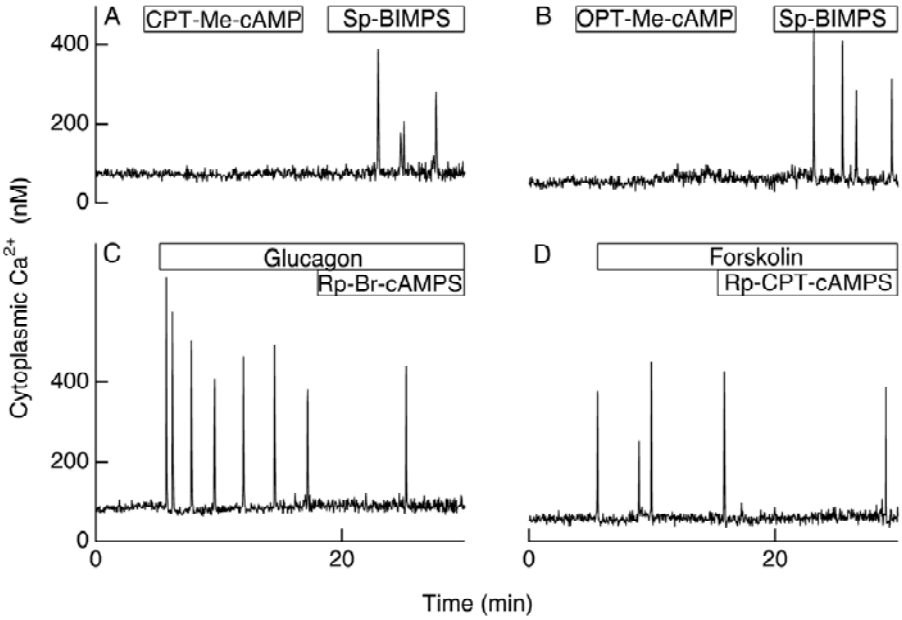
A
CPT-Me-cAMP
Sp-BIMPS
B
OPT-Me-cAMP
Sp-BIMPS
C
Glucagon
Rp-Br-cAMPS
D
Forskolin
Rp-CPT-cAMPS
Cytoplasmic Ca2+ (nM)
400
200
0
0
20
Time (min)

FIG. 3

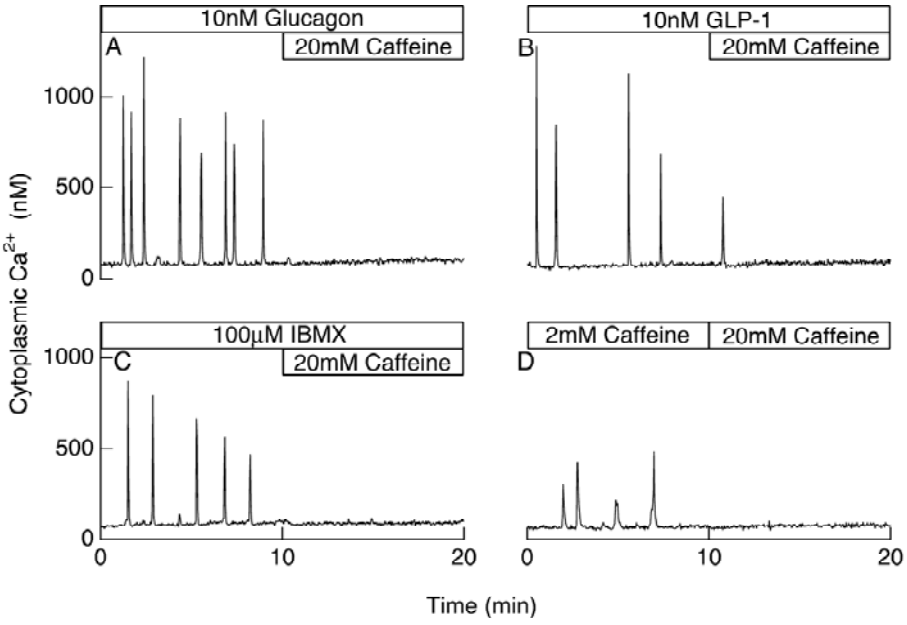
10nM Glucagon
20mM Caffeine
A
10nM GLP-1
20mM Caffeine
B
100µM IBMX
20mM Caffeine
C
2mM Caffeine
20mM Caffeine
D
Cytoplasmic Ca2+ (nM)
1000
500
0
0
10
20
Time (min)

FIG. 4

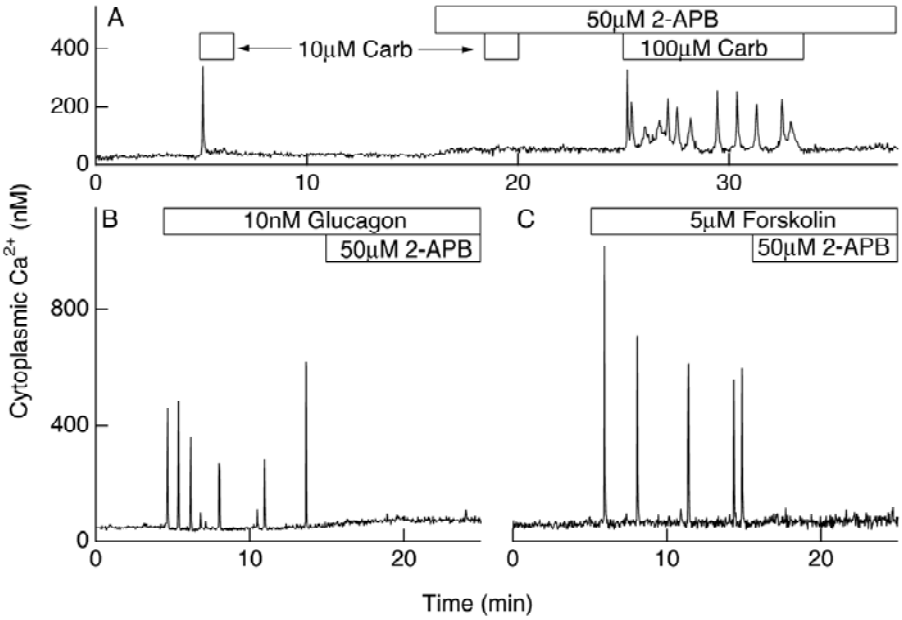
A
50μM 2-APB
10μM Carb
100μM Carb
400
200
0
0
10
20
30
B
10nM Glucagon
50μM 2-APB
C
5μM Forskolin
50μM 2-APB
800
400
0
0
10
20
0
10
20
Cytoplasmic Ca2+ (nM)
Time (min)

FIG. 5

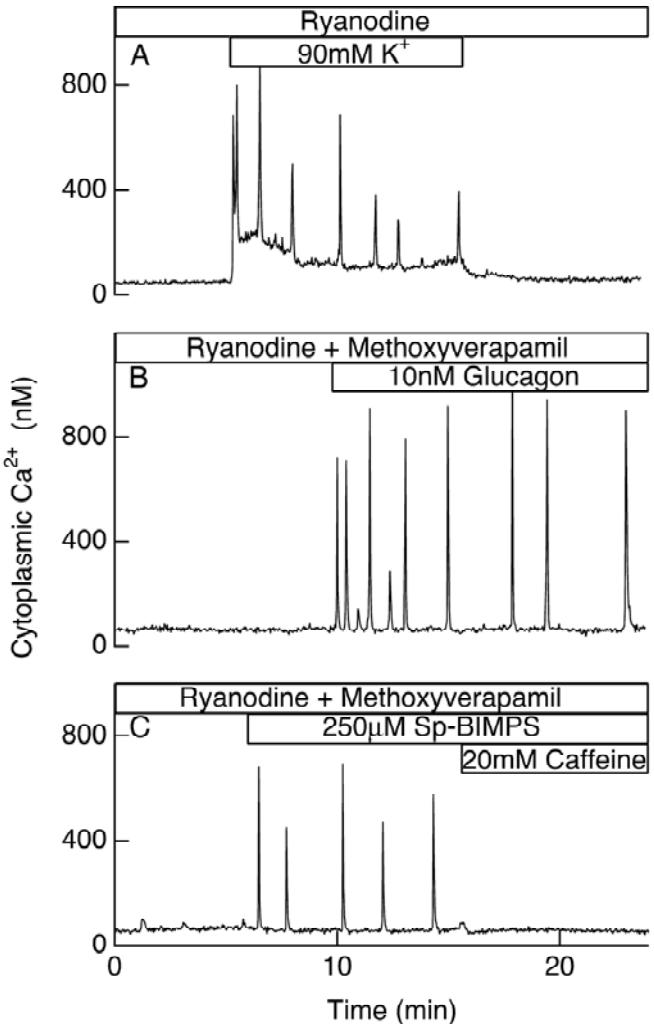
Ryanodine
A
90mM K+
800
400
0
Ryanodine + Methoxyverapamil
B
10nM Glucagon
800
400
0
Cytoplasmic Ca2+ (nM)
Ryanodine + Methoxyverapamil
C
250μM Sp-BIMPS
20mM Caffeine
800
400
0
0
10
20
Time (min)

FIG. 6

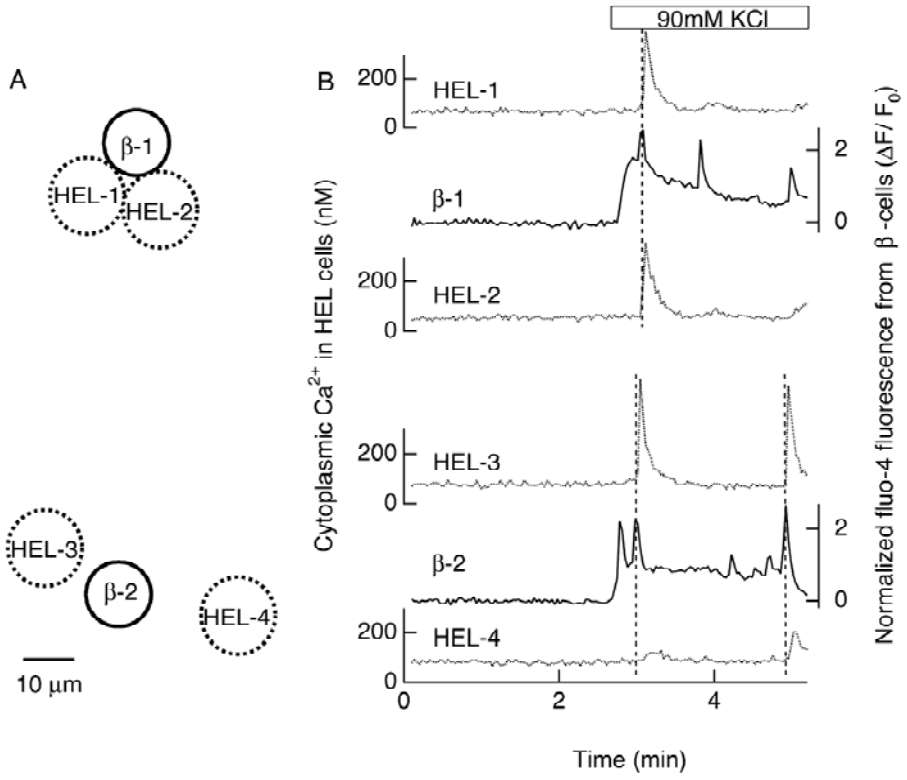
A
β-1
HEL-1
HEL-2
HEL-3
β-2
HEL-4
10 μm
B
90mM KCl
HEL-1
β-1
HEL-2
HEL-3
β-2
HEL-4
200
0
2
0
0
2
4
Time (min)
Cytoplasmic Ca2+ in HEL cells (nM)
Normalized fluo-4 fluorescence from β -cells (ΔF/ F0)

FIG. 7

[1] The abbreviations used are K_{ATP} channel, ATP/ADP-sensitive K^+ channel; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; RyR, ryanodine receptor; CICR, Ca^{2+}-induced Ca^{2+} release; PKA, protein kinase A; Epac, cAMP-regulated guanine nucleotide exchange factor; fura-2/AM), fura-2 acetoxymethyl ester; fluo-4/AM, fluo-4 acetoxymethyl; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate; 8-pCPT-2'-O-Me-cAMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate; 8-pMeOPT-2'-O-Me-cAMP, 8-(4-methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclicmonophosphate; Sp-5,6-DCl-cBIMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate, Sp-isomer; Rp-8-Br-cAMPS, 8- bromoadenosine- 3', 5'- cyclic monophosphorothioate, Rp-isomer; Rp-8-CPT-cAMPS, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer; GLP-1, human glucagon-like peptide-1 amide fragment 7-36, 8-Br-cAMP, 8-bromoadenosine-3',5'- cyclic monophosphate; 8-Br-cGMP, 8-bromoguanosine-3',5'-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; CPA, cyclopiazonic acid; 2-APB, 2-aminoethoxydiphenyl borate; PKG, protein kinase G; SERCA, sarco(endo)plasmic reticululum ATPase.