cAMP-regulated guanine nucleotide exchange factor II (Epac2) mediates Ca^{2+}-induced Ca^{2+} release in INS-1 pancreatic β-cells

Guoxin Kang, Oleg G. Chepurny and George G. Holz

Department of Physiology and Neuroscience, New York University School of Medicine, New York, NY 10016, USA

(Resubmitted 23 April 2001; accepted after revision 21 June 2001)

1. The signal transduction pathway responsible for cAMP-dependent Ca^{2+}-induced Ca^{2+} release (CICR) from endoplasmic reticulum Ca^{2+} stores was assessed in the insulin-secreting cell line INS-1.

2. CICR was triggered by the GLP-1 receptor agonist exendin-4, an effect mimicked by caffeine, Sp-cAMPS or forskolin. CICR required influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels, and was blocked by treatment with nimodipine, thapsigargin, or ryanodine, but not by the IP_3 receptor antagonist xestospongin C.

3. Treatment with the cAMP antagonist 8-Br-Rp-cAMPS blocked CICR in response to exendin-4, whereas the PKA inhibitor H-89 was ineffective when tested at a concentration demonstrated to inhibit PKA-dependent gene expression.

4. RT-PCR of INS-1 cells demonstrated expression of mRNA coding for the type-II isoform of cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF-II, Epac2).

5. CICR in response to forskolin was blocked by transient transfection and expression of a dominant negative mutant isoform of cAMP-GEF-II in which inactivating mutations were introduced into the exchange factor's two cAMP-binding domains.

6. It is concluded that CICR in INS-1 cells results from GLP-1 receptor-mediated sensitization of the intracellular Ca^{2+} release mechanism, a signal transduction pathway independent of PKA, but which requires cAMP-GEF-II.

The mobilization of Ca^{2+} from endoplasmic reticulum (ER) Ca^{2+} stores is now recognized as playing a highly significant role in the regulation of multiple cellular functions. Gating of inositol trisphosphate receptor (IP_3-R) or ryanodine receptor (RYR) intracellular Ca^{2+} release channels produces an increase of $[Ca^{2+}]_i$ that serves as a stimulus for induction of gene expression, mitochondrial metabolism, excitation–contraction coupling, and hormone or neurotransmitter secretion (Berridge, 1998). Ca^{2+} is mobilized from IP_3-R- or RYR-regulated Ca^{2+} stores by a process referred to as Ca^{2+}-induced Ca^{2+} release (CICR; Bootman & Berridge, 1995; Berridge, 1996, 1997). CICR results from the stimulatory action of Ca^{2+} at Ca^{2+} release channels, thereby initiating a regenerative Ca^{2+} wave propagating as a transient, and in some cases oscillatory increase of $[Ca^{2+}]_i$. Although the mobilization of Ca^{2+} from IP_3-R-regulated Ca^{2+} stores is recognized as being of general importance for both excitable and non-excitable cells, the functional significance of CICR originating from RYR-regulated Ca^{2+} stores is only now becoming more fully understood.

The physiological importance of RYR-regulated CICR was first established in studies of cardiac myocyte excitation–contraction coupling where influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (VDCCs) was demonstrated to stimulate the opening of RYR Ca^{2+} release channels (Fabiato, 1983; Nabauer *et al.* 1989; Niggli, 1999). More recently, it has been suggested that endocrine cells and neurons also utilize RYR-regulated CICR as a means of mobilizing Ca^{2+} from Ca^{2+} stores (Islam *et al.* 1992, 1998; Leech *et al.* 1994; Gromada *et al.* 1995; Holz *et al.* 1999; Usachev & Thayer, 1999). In some cell types, this can lead to a stimulation of exocytosis (Cheek *et al.* 1993; von Ruden & Neher, 1993; Tse *et al.* 1993, 1997; Guo *et al.* 1996; Narita *et al.* 1998, 2000).

To explore what role RYR-dependent CICR might play as a stimulus for insulin secretion, we initiated studies utilizing the insulinotropic hormone glucagon-like peptide-1 (GLP-1; Drucker, 1998; Kieffer & Habener, 1999). GLP-1 promotes pancreatic β-cell depolarization by inhibiting ATP-sensitive K^+ (K-ATP) channels (Holz & Habener, 1992; Holz *et al.* 1993), and it also facilitates

insulin secretion via a more direct effect at the exocytotic secretory apparatus (Gromada *et al.* 1998*b*,*c*). An unusual aspect of GLP-1 signal transduction is its ability to mobilize β-cell Ca^{2+} stores in a cAMP-dependent manner (Gromada *et al.* 1995; Holz *et al.* 1999). The relevant Ca^{2+} release channels targeted by GLP-1 are most likely to correspond to RYR-2, the type-2 isoform of ryanodine receptor (Islam *et al.* 1998; Holz *et al.* 1999), and all available evidence indicates that GLP-1 sensitizes RYR-2 to stimulatory effects of cytosolic Ca^{2+}, thereby increasing the likelihood that such channels will open in response to Ca^{2+} (Gromada *et al.* 1995; Holz *et al.* 1999).

Exactly which signal transduction pathway explains how GLP-1 triggers CICR has remained uncertain. GLP-1 stimulates cAMP synthesis (Thorens, 1992; Drucker, 1998), but has only a weak stimulatory effect on IP_3 production in β-cells (Zawalich *et al.* 1993; Zawalich & Zawalich, 1996). However, GLP-1 does stimulate IP_3 production in COS cells and *Xenopus* oocytes expressing recombinant GLP-1 receptors (Wheeler *et al.* 1993; Gromada *et al.* 1998*a*). GLP-1 also activates protein kinase A (PKA; Lester *et al.* 1997), Ca^{2+}–calmodulin-dependent protein kinase (Susini *et al.* 2000), phosphatidylinositol 3-kinase (PI-3K; Buteau *et al.* 1999) and mitogen-activated protein kinases (MAPK; Frodin *et al.* 1995; Montrose-Rafizadeh *et al.* 1999).

The cAMP-dependent action of GLP-1 may not be limited to its effects mediated by PKA. A newly discovered family of cAMP-binding proteins has been characterized, molecules referred to as cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs, also known as Epac; Kawasaki *et al.* 1998; de Rooij *et al.* 1998; Leech *et al.* 2000). The cAMP-GEFs interact with the Ras-related small G protein Rap1 to initiate a cascade of protein kinase-mediated phosphorylation reactions catalysed by B-Raf and MAPK. Furthermore, the interaction of cAMP-GEFs with the Rab3 small G protein effector Rim is reported to stimulate exocytosis of insulin in β-cells (Ozaki *et al.* 2000). Therefore, there is reason to believe that activation of cAMP-GEFs might also explain, at least in part, how CICR in β-cells is triggered in response to agents (forskolin, 8-Br-cAMP, Sp-cAMPS) that produce an increase of $[cAMP]_i$ analogous to that produced by GLP-1.

Here we examined what role cAMP-GEFs may play as mediators of CICR in the insulin-secreting cell line INS-1. The INS-1 cells are a radiation-induced rat insulinoma that respond to glucose and GLP-1 (Asfari *et al.* 1992; Skoglund *et al.* 2000), and were previously characterized as a model system in which to investigate RYR-mediated CICR (Gamberucci *et al.* 1999). Measurements of $[Ca^{2+}]_i$ were obtained under conditions in which wild-type or mutant isoforms of cAMP-GEFs were introduced by transient transfection. We report that RYR-regulated CICR is initiated, at least in part, by a PKA-independent signalling mechanism that most probably involves cAMP-GEF-II. Some of these finding have been reported in preliminary form (Kang & Holz, 2000, 2001).

METHODS

Cell culture

INS-1 cells (passages 70–90) were obtained from Dr Maryam Asfari (Asfari *et al.* 1992). Cell cultures were maintained in a humidified incubator (95% air–5% CO_2) at 37 °C in RPMI 1640 culture medium containing 10 mM Hepes, 11.1 mM glucose, 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate and 50 μM 2-mercaptoethanol. INS-1 cells were passaged by trypsinization and subcultured once a week. Cell suspensions were plated on glass coverslips (25CIR-1; Fisher Scientific, Chicago, IL, USA) coated with 1 mg ml^{-1} concanavalin A (type V; Sigma Chemical Co.), which facilitates adherence of cells to glass surfaces. All reagents for cell culture were obtained from Life Technologies (Rockville, MD, USA), and all experiments illustrated were performed in triplicate after a 16–36 h equilibration in culture medium in order to assure reproducibility.

Transfection of INS-1 cells for overexpression of wild-type and mutant cAMP-GEF-II

Wild-type cAMP-GEF-II (GenBank accession number AB021132) expression vector pSRα-cAMP-GEFII and mutant cAMP-GEF-II expression vector pSRα-cAMP-GEFII (G114E/G422D) were provided by Dr Susumu Seino (Chiba University, Japan; Ozaki *et al.* 2000). The mutant cAMP-GEF-II acts in a dominant negative manner because it contains inactivating G114E and G422D amino acid substitutions in the A and B cAMP-binding domains of the exchange factor, respectively. INS-1 cell cultures grown to 80% confluence in 35 mm tissue culture dishes were subjected to transient transfection using 1 μg cAMP-GEF-II expression vector reconstituted in Lipofectamine Plus reagent (Life Technologies). At 24 h post-transfection, the cells were lifted by trypsinization, plated onto glass coverslips, and maintained in normal INS-1 cell culture medium. Experiments were then performed 48 h post-transfection. Transfection efficiency was estimated to be 10–20%, as determined by use of an enhanced yellow fluorescent protein (EYFP) expression vector.

RIP2–EYFP for fluorescence-based selection of transfected cells in culture

In order to positively identify transfected INS-1 cells, the transfection cocktail containing 1 μg of cAMP-GEF-II expression vector was supplemented with 100 ng of a plasmid in which expression of EYFP was placed under the control of the rat insulin gene II promoter (RIP2). Expression of EYFP was achieved by replacement of a CMV promoter within the pEYFP-N1 expression vector (Clonetech, Palo Alto, CA, USA) for a −700 bp *Bam*HI fragment of RIP2. EYFP fluorescence was monitored in living cells 2 days post-transfection using 513 nm excitation and 527 nm emission filters (Fig. 1*A*). Once an EYFP-positive cell was identified as having been transfected, the filter set was manually switched to a fura-2 filter set allowing ratiometric determinations of $[Ca^{2+}]_i$ (Fig. 1*A*; see below). The spectral properties of EYFP do not interfere with fura-2 based measurements of $[Ca^{2+}]_i$ (Fig. 1*B*; Kang & Holz, 2000), and less than 1% crossover exists between fura-2 and EYFP when using filter sets selective for each probe (G. G. Holz, unpublished observations). Control experiments demonstrated that overexpression of EYFP in INS-1 cells does not interfere with KCl- or glucose-dependent Ca^{2+} signalling, nor does it interfere with glucose-dependent insulin gene promoter activity (G. G. Holz, unpublished observations).

Measurement of $[Ca^{2+}]_i$

The fura-2 loading solution consisted of standard extracellular saline (SES) containing (mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes and 5.6 D-glucose. The pH was adjusted to 7.35 with NaOH

and the osmolarity was adjusted to 295 mosmol l^{-1} using H_2O. The SES was supplemented with 1 μM fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes Inc., Eugene, OR, USA), 2% FBS and 0.02% Pluronic F-127 (w/v; Molecular Probes Inc.). Cells were exposed to the fura-2 AM-containing solution for 20–30 min at 22°C. The loading solution was then removed, and cells were equilibrated in fresh SES for 10 min at 22°C. Under these conditions, we previously demonstrated that 9% of fura-2 is compartmentalized, the remaining 91% being restricted to the cytosol (Leech *et al.* 1994). Experiments were performed at 32°C using an Eclipse TE300 inverted microscope (Nikon, Melville, NY, USA) outfitted with a temperature-controlled stage (Medical Systems Corp., Greenvale, NY, USA), a superfusion system, and a ×100 Nikon UVF oil immersion objective (NA = 1.3). Dual excitation wavelength microspectrofluorimetry was performed ratiometrically at 0.5 s intervals using a digital video imaging system outfitted with an intensified CCD camera (IonOptix Corp., Milton, MA, USA). A rotating chopper mirror delivered excitation light at 340 or 380 nm, and emitted light was measured at 510 nm (Fig. 1*A*). The average of five video frames of imaging data was used to calculate numerator and denominator values for determination of 340 nm/380 nm ratio values. The $[Ca^{2+}]_i$ was calculated according to established methods (Grynkiewicz *et al.* 1985) as:

$$[Ca^{2+}]_i = K_d \beta[(R - R_{min})/(R_{max} - R)], \quad (1)$$

where R is the experimentally derived fluorescence ratio, and R_{min} and R_{max} are ratios measured using calibration solutions containing no Ca^{2+} or saturating Ca^{2+}, respectively. K_d is the dissociation constant (224 nM) describing the interaction of fura-2 and Ca^{2+}, and β is the fluorescence ratio of free/bound fura-2 measured with 380 nm excitation. Calibration of the raw fluorescence values was performed using fura-2 pentapotassium salt dissolved in calibration buffers from Molecular Probes Inc. (Calcium Calibration Kit 1 with Mg^{2+}). Values of R_{min} and R_{max} were 0.20 and 7.70 for the experiments described.

Sources of reagents and preparation of test solutions

GLP-1-(7–37), GLP-1-(7–36)-amide, exendin-4, exendin-(9–39), caffeine, thapsigargin, nimodipine, forskolin, H-89 and ryanodine were from Sigma. 8-Br-Rp-cAMPS and Sp-cAMPS were from BioLog Life Sciences Institute (Bremen, Germany). Xestospongin C was from Calbiochem (La Jolla, CA, USA). Test solutions were dissolved in SES prior to each experiment. For studies examining effects of peptides, the SES also contained 0.05% human serum albumin (HSA, fraction V; Sigma) to protect against their binding to glass or plastic surfaces. No effect of HSA itself was observed. Test solutions were applied to individual cells from 'puffer' micropipettes using a PicoSpritzer II pressure ejection system (General Valve Corp., NJ, USA; Holz *et al.* 1993).

pCRE-Luc reporter gene assay

The pCRE-Luc plasmid (Stratagene, La Jolla, CA, USA) introduced into transformed JM109 cells was prepared using the Wizard Midi Prep procedure (Promega Inc., Madison, WI, USA). INS-1 cells were harvested as a single cell suspension, and Lipofectamine Plus containing 1 μg pCRE-Luc was added according to the manufacturer's protocol (Life Technologies). Cells were then pipetted onto 96-well culture plates (Costar, Fisher Scientific). Two days post-transfection, cells were lysed and assayed at 37°C for luciferase activity using a Luciferase Assay Kit (Tropix, Bedford, MA, USA) in conjunction with a plate reading luminometer allowing automated injection of ATP and luciferin solutions (Model TR-717, Applied Biosystems, Foster City, CA, USA). Experiments were carried out in triplicate, and statistical analysis was performed using the ANOVA test combined with Fisher's PLSD test.

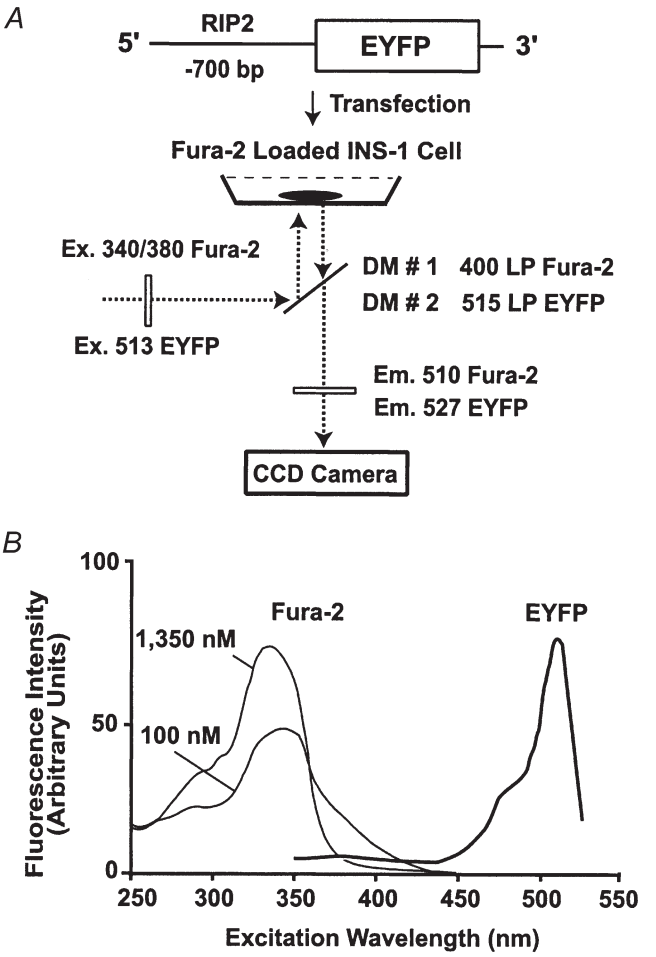

Figure 1. Fluorescence microscopy allows co-detection of EYFP and fura-2 in single living INS-1 cells

A, optical components of the imaging system. RIP2–EYFP was introduced into INS-1 cells by transient transfection, and cells were loaded with fura-2 by incubation in fura-2 AM-containing SES. For detection of EYFP, a xenon arc lamp delivered excitation light (Ex.) filtered at 513 nm and reflected by a 515 nm long pass (LP) dichroic mirror (DM). EYFP epifluorescence passed through a 527 nm emission filter (Em.) for detection by an intensified CCD camera interfaced with a digital video imaging system. For detection of fura-2, a rotating chopper mirror delivered excitation light of 340 or 380 nm to a second filter set in which was housed a 400 nm long pass dichroic mirror and a 510 nm emission filter. *B*, excitation spectra for EYFP (bold line) and fura-2 at two different concentrations of free Ca^{2+} (100 or 1350 nM). Little or no excitation of EYFP occurs in response to the 340 or 380 nm excitation light used for fura-2, and conversely, little or no excitation of fura-2 occurs in response to light delivered at the excitation maximum (513 nm) for EYFP.

Analysis of cAMP-GEF mRNA expression by RT-PCR

Total RNA was prepared from INS-1 cells by the TRIzol method (Life Technologies), and cDNA synthesis was catalysed by AMV reverse transcriptase using oligo d(T) primers (Reverse Transcription System, Promega). A cDNA synthesis reaction to which no reverse transcriptase was added served as a negative control. The design of PCR primers was based on the published sequence for rat cAMP-GEF-II (Kawasaki *et al.* 1998; accession number U78517). Optimal PCR primers were selected using a computer-based analysis program (Lasergene 5.0, DNASTAR Inc., Madison, WI, USA). The cDNA was amplified using *Taq* DNA polymerase (TaKaRa Biologicals, Shiga, Japan). The thermal cycling parameters consisted of an initial denaturation step for 5 min at 95 °C, followed by 20 cycles consisting of: 30 s at 95 °C, 60 s at 57 °C and 90 s at 72 °C. The final extension step was for 5 min at 72 °C. PCR products were resolved by 1 % agarose gel electrophoresis, and DNA was stained with ethidium bromide for fluorescence detection. PCR products were extracted from agarose using Quiaex 2 (Quiagen, Inc., Valencia, CA, USA), and ligated into the pCR4TOPO vector (Invitrogen, San Diego, CA, USA) for transformation of OneShot cells (Invitrogen). Transformants were isolated by antibiotic resistance selection, and midiprep plasmid DNA was prepared (Wizard DNA Purification Kit, Promega) and sequenced for verification of PCR product identity.

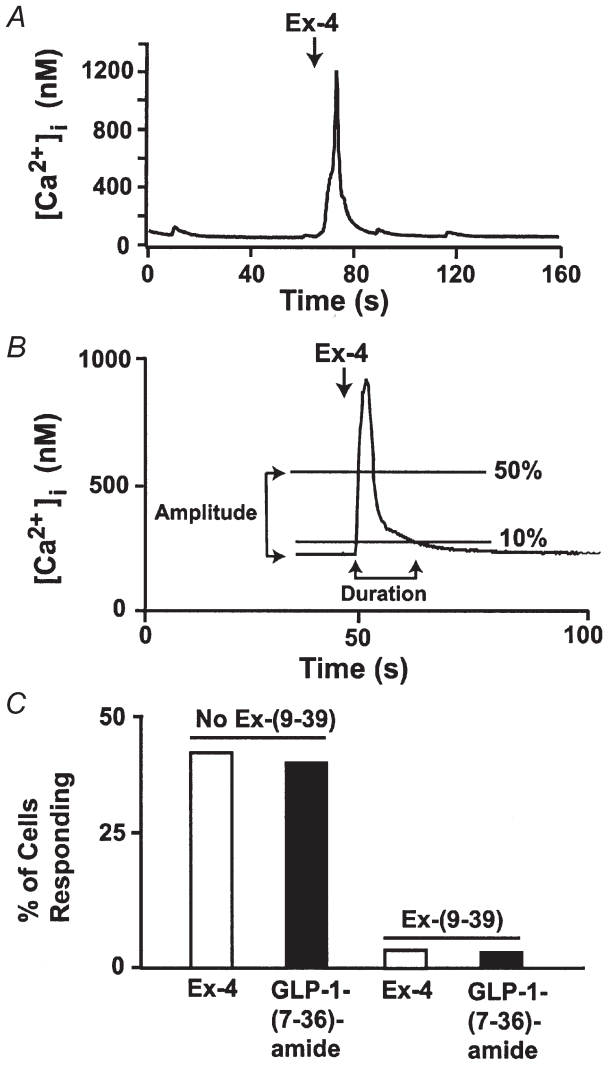

Figure 2. Elevation of $[Ca^{2+}]_i$ by GLP-1 receptor agonist exendin-4 in INS-1 cells

A, local application of 10 nM exendin-4 (Ex-4) produced a transient increase of $[Ca^{2+}]_i$. *B*, the response to exendin-4 was defined as a transient increase of $[Ca^{2+}]_i$, the duration of which did not exceed 30 s when measured at the 10 % response amplitude cut-off, and the amplitude of which exceeded 200 nM when measured at the 50 % cut-off. *C*, population study demonstrating that CICR in response to 1 nM exendin-4 or 1 nM GLP-1-(7–36)-amide was blocked by prior exposure of INS-1 cells to 1 μM of the GLP-1 receptor antagonist exendin-(9–39). Experiments were performed using INS-1 cells equilibrated in saline containing 11.1 mM glucose, here and in subsequent figures. Arrows in *A* and *B* indicate a 30 s application of exendin-4 using a puffer pipette.

RESULTS

Exendin-4 stimulates an increase of $[Ca^{2+}]_i$ in INS-1 cells

INS-1 cells loaded with fura-2 responded to the GLP-1 receptor agonist exendin-4 by exhibiting a transient increase of $[Ca^{2+}]_i$ (Fig. 2*A*). The response to exendin-4 exhibited an inflection on the rising phase of the Ca^{2+} spike, indicative of an initial increase of $[Ca^{2+}]_i$ that triggered a regenerative response after a short delay. This action of exendin-4 was quantified by measuring the response amplitude and duration at the 50 % and 10 % cut-off values, respectively (Fig. 2*B*). The mean response amplitude was 1100 ± 150 nM, and the mean response duration was 14 ± 3 s. The action of exendin-4 reported here is analogous to the previously reported stimulatory effects of GLP-1 on $[Ca^{2+}]_i$ in rat pancreatic β-cells (Holz *et al.* 1999). It is mediated by GLP-1 receptors because the action of 1 nM exendin-4 was reproduced by 1 nM of the GLP-1 receptor agonist GLP-1-(7–36)-amide, but blocked by 1 μM of the receptor antagonist exendin-(9–39) (Fig. 2*C*).

Pharmacological properties of CICR triggered by exendin-4

To validate that the fast transient increase of $[Ca^{2+}]_i$ observed in response to exendin-4 did in fact result from CICR, we performed pharmacological studies of Ca^{2+} signalling in INS-1 cells. Dose–response analysis (Fig. 3*A*) demonstrated that exendin-4 stimulated CICR over a concentration range of 0.1–10 nM of the peptide (EC_{50} 0.8 nM). The action of exendin-4 to stimulate CICR was reproduced by caffeine (Fig. 3*A*, see also Fig. 4*A*), as well as by the cAMP-elevating agent forskolin (Figs 3*A* and 4*B*), or by the cAMP agonist Sp-cAMPS (Fig. 3*A*). In agreement with the previously reported action of GLP-1 in stimulating CICR in rat β-cells (Holz *et al.* 1999), the actions of exendin-4 and forskolin in this assay were blocked by pretreatment of INS-1 cells with the L-type VDCC antagonist nimodipine (1 μM), or by pretreatment with the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 μM; Fig. 3*B*

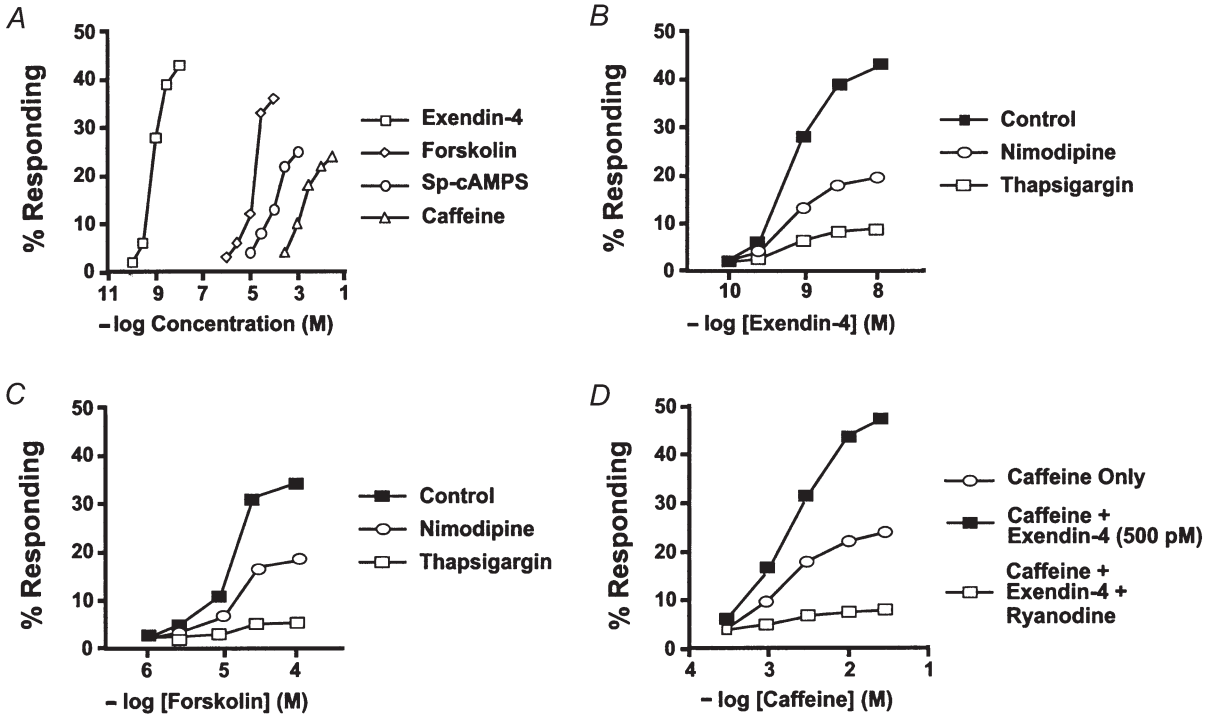

Figure 3. Pharmacological properties of Ca^{2+} signalling in INS-1 cells

A, dose–response analysis illustrating the concentration range over which exendin-4, forskolin, Sp-cAMPS, and caffeine stimulated CICR. Such 'all or nothing' responses were quantified by determining the percentage of cells exhibiting a transient increase of $[Ca^{2+}]_i$, as defined in Fig. 2*B*. *B* and *C*, CICR initiated by exendin-4 or forskolin was suppressed by nimodipine (1 μM) and by thapsigargin (1 μM). *D*, CICR in response to caffeine was potentiated by a low concentration of exendin-4 (500 pM). The synergistic interaction of caffeine and exendin-4 was blocked by pretreatment of cells for 30 min in a solution containing 5 mM caffeine and 1 μM ryanodine.

and *C*). Of particular interest was the observation that there existed a synergistic interaction of exendin-4 and caffeine to trigger CICR (Fig. 3*D*). This interaction was eliminated by a 30 min pretreatment of INS-1 cells with a solution containing 5 mM caffeine and 1 μM ryanodine (Fig. 3*D*). The stimulation of CICR by caffeine was not simply a secondary consequence of this methylxanthine's known inhibitory effect on cAMP phosphodiesterase. The

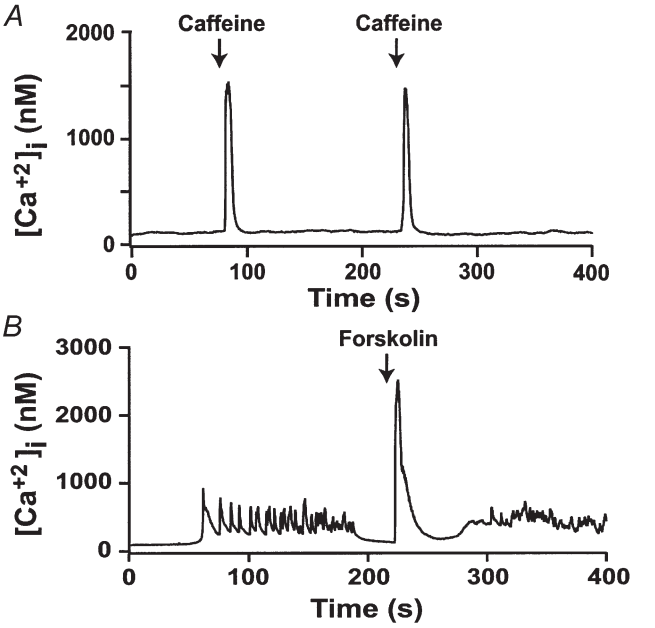

Figure 4. Pharmacological stimulation of CICR by caffeine and forskolin in INS-1 cells

A, caffeine was applied twice at a concentration of 10 mM. *B*, forskolin was applied once at a concentration of 10 μM. Note that prior to administration of forskolin, this cell exhibited spontaneous oscillation of $[Ca^{2+}]_i$. Arrows in *A* and *B* indicate a 10 s application of each test substance.

Table 1. RT-PCR of INS-1 cell mRNA for cAMP-GEF-II (GenBank U78517)

Primer pair	Nucleic acid sequence	Sequence amplified	Predicted size (bp)	Detected in INS-1?
Forward no. 1	5′ CAGAACGGTGCGCTACTACAGGA 3′	716–738	408	Yes
Reverse no. 1	5′ TGCCAACCGTGAAGAAGATGACTA 3′	1100–1123	—	—
Forward no. 2	5′ GTGGGGACGTTTGAACTGATGAGC 3′	135–158	440	Yes
Reverse no. 2	5′ AGCCTGTACGCCTTGTGATTTCTG 3′	551–574	—	—
Forward no. 3	5′ GCCAGAACGGTGCGCTACTACA 3′	714–735	595	Yes
Reverse no. 3	5′ ACCCTCCCCAGAACCCAGACC 3′	1288–1308	—	—

RT-PCR was performed using 3 different PCR primer pairs complementary to the 3′ terminus of the rat cAMP-GEF-II coding sequence, and extending into the 3′-untranslated region.

action of caffeine was not blocked by pretreatment with the cAMP antagonist 8-Br-Rp-cAMPS (Fig. 5*A*).

A role for cAMP in support of CICR triggered by exendin-4

It has been suggested that cAMP-regulated, PKA-dependent phosphorylation sensitizes RYR-2 to stimulatory effects of cytosolic Ca^{2+}, thereby facilitating CICR (Gromada *et al.* 1995; Holz *et al.* 1999). Consistent with this concept, CICR in response to the cAMP-elevating agent forskolin was unaffected by the membrane-permeant IP_3 receptor antagonist xestospongin C (Fig. 5*B*).

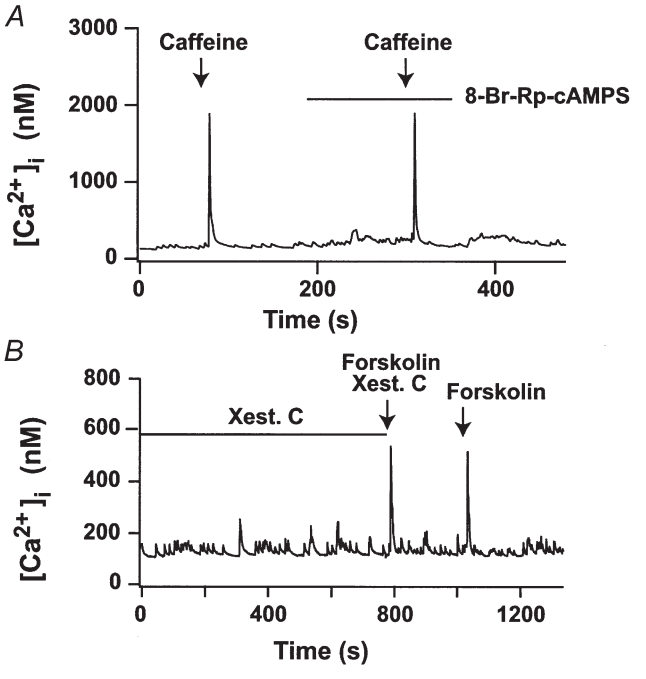

Figure 5. Analysis of the effects of 8-Br-Rp-cAMPS and xestospongin C

A, CICR in response to a 10 s application of 10 mM caffeine (arrows) was not blocked by pretreatment with 200 μM of the cAMP antagonist 8-Br-Rp-cAMPS. *B*, CICR in response to a 10 s application of 10 μM forskolin (arrows) was not blocked by pretreatment with 20 μM of the IP_3 receptor antagonist xestospongin C.

As expected, pretreatment of INS-1 cells with the cAMP antagonist 8-Br-Rp-cAMPS (200 μM) inhibited the action of exendin-4 in this assay (Fig. 6*A*). To investigate whether exendin-4 exerted its stimulatory action via PKA, a well-characterized PKA antagonist (H-89) was

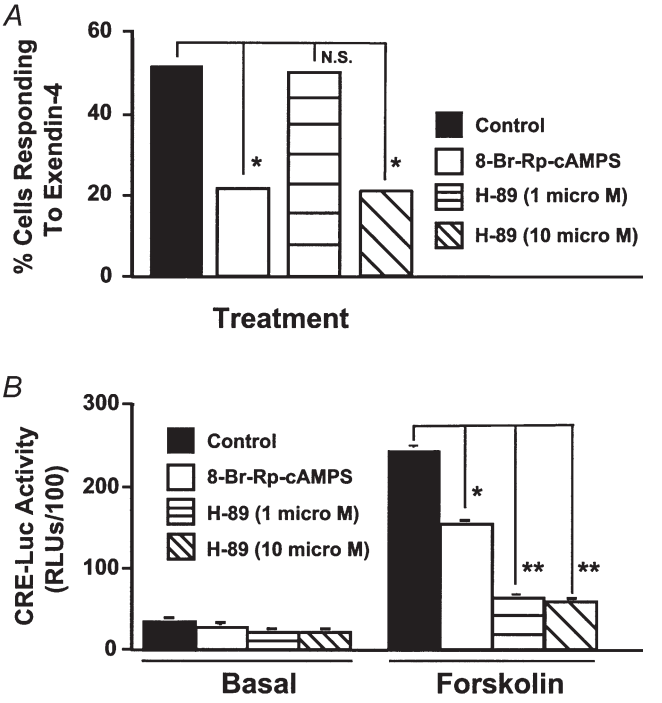

Figure 6. Antagonist properties of 8-Br-Rp-cAMPS and H-89 assessed in INS-1 cells

A, the cyclic AMP antagonist 8-Br-Rp-cAMPS (200 μM) inhibited CICR measured in response to a 30 s application of exendin-4 (10 nM). The PKA inhibitor H-89 did not block CICR in response to exendin-4 when tested at a concentration of 1 μM, but was an effective blocker when tested at 10 μM. *B*, CRE-dependent expression of luciferase in transfected INS-1 cells was stimulated by a 4 h exposure to forskolin (2 μM), and was partially inhibited by 8-Br-Rp-cAMPS (200 μM). Note that the action of forskolin was inhibited by a low (1 μM) as well as a high (10 μM) concentration of H-89. $*P < 0.005$, $**P < 0.001$, relative to Control.

tested. We found that the action of exendin-4 was not blocked by pretreatment with 1 μM H-89 (Fig. 6*A*), a concentration that allows selective inhibition of PKA (Hidaka & Kobayashi, 1992). A 10-fold higher concentration of H-89 reduced the action of exendin-4 (Fig. 6*A*), but also produced a generalized suppression of spontaneous Ca^{2+} oscillations (data not shown). This effect is most likely to be due to non-selective inhibition of a broad spectrum of protein kinases (Hidaka & Kobayashi, 1992; Bode *et al.* 1999).

The efficacy and specificity of 8-Br-Rp-cAMPS and H-89 were assessed in a luciferase-based reporter assay considered to be diagnostic for cAMP and PKA signalling (Skoglund *et al.* 2000). INS-1 cells were transfected with CRE-Luc, a plasmid in which expression of luciferase is directed by a promoter containing multimerized synthetic cAMP response elements (CREs). Cells were then assayed for luciferase activity following a 4 h exposure to 2 μM forskolin. Under these conditions, CRE-Luc activity was stimulated by forskolin (Fig. 6*B*), an effect inhibited by treatment with 8-Br-Rp-cAMPS (200 μM) or H-89 (1 μM). Therefore, 8-Br-Rp-cAMPS and H-89 are effective antagonists of cAMP and PKA signalling in INS-1 cells when used at low concentrations, and when tested within the appropriate experimental context. Taken together, this analysis indicates that CICR in response to exendin-4 results from activation of a cAMP signalling pathway that is relatively insensitive to H-89, and which might be, at least in part, independent of PKA. Consistent with this concept, RT-PCR analysis of INS-1 cells demonstrated expression of mRNA coding for rat cAMP-GEF-II (Table 1). Nucleic acid sequence analysis of these PCR products demonstrated them to be 100% identical to cAMP-GEF-II (data not shown).

Downregulation of CICR by expression of dominant negative cAMP-GEF-II

We next sought to more directly demonstrate a role for cAMP-GEF-II as a mediator of CICR by use of a molecular biological approach employing wild-type or dominant negative isoforms of the exchange factor. The dominant negative cAMP-GEF-II is a mutant isoform in which inactivating point mutations have been introduced into the two cAMP-binding domains (Ozaki *et al.* 2000). The cAMP-GEF-II cDNA was introduced by transient transfection in conjunction with use of RIP2–EYFP as a marker allowing positive identification of transfected INS-1 cells (Methods, Fig. 1). A population study was then conducted in order to assess the extent to which cAMP-GEF-II mediates CICR in response to forskolin. Cells not transfected exhibited the typical transient increase of $[Ca^{2+}]_i$ when challenged with 10 μM forskolin (Fig. 7*A*), and this response matched closely the pattern of CICR observed in cells transfected with wild-type cAMP-GEF-II (Fig. 7*B*). However, when cells were transfected with dominant negative cAMP-GEF-II, the action of forskolin was largely abrogated, and only a small,

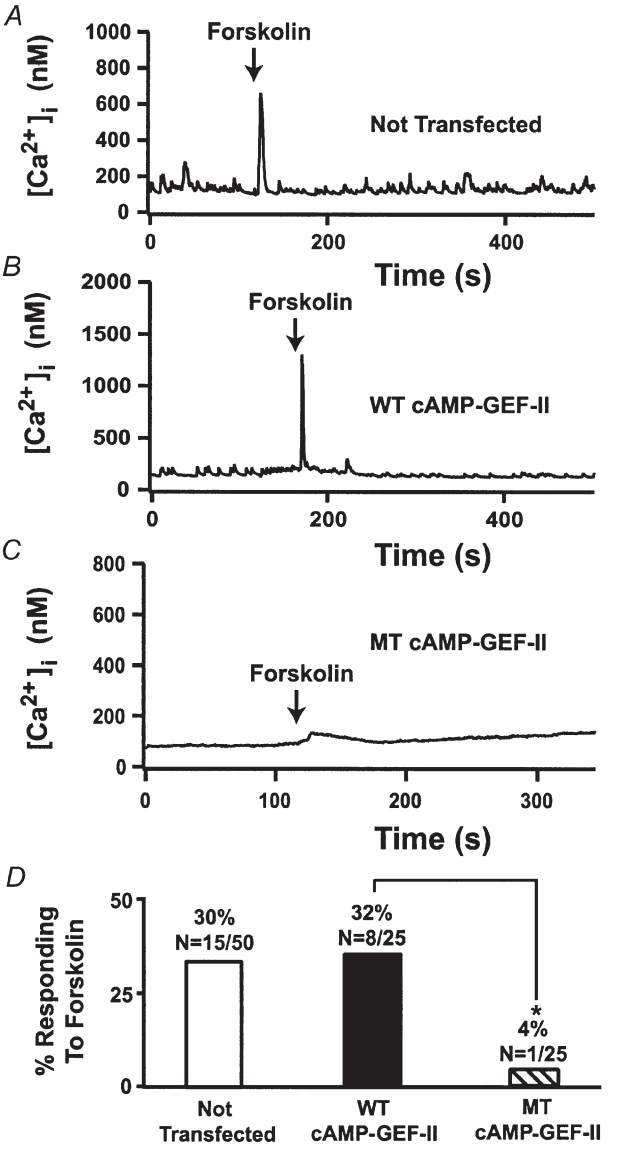

Figure 7. Inhibition of forskolin-stimulated CICR by dominant negative cAMP-GEF-II

Single cell suspensions of INS-1 cells were treated with Lipofectamine Plus supplemented with RIP2–EYFP and wild-type or dominant negative cAMP-GEF-II expression vectors. Cells were then plated on glass coverslips for loading with fura-2 and measurement of $[Ca^{2+}]_i$. *A*, untransfected cells not exhibiting EYFP fluorescence responded to application of forskolin by generating CICR. *B*, CICR in response to forskolin was also observed in transfected cells expressing EYFP and wild-type (WT) cAMP-GEF-II. *C*, CICR in response to forskolin was markedly inhibited in transfected cells expressing EYFP and mutant (MT) dominant negative cAMP-GEF-II. A residual response to forskolin was sometimes observed in these cells. It consisted of a small sustained increase of $[Ca^{2+}]_i$. *D*, population study demonstrating antagonism of forskolin-induced CICR by dominant negative cAMP-GEF-II. * $P \leqslant 0.001$, Student's *t* test. Arrows in *A*–*C* indicate application of 10 μM forskolin for 10 s.

sustained increase of $[Ca^{2+}]_i$ was sometimes observed (Fig. 7*C*). These findings are summarized in the bar graph presented in Fig. 7*D*.

DISCUSSION

A role for cAMP-GEF-II as a mediator of CICR

CICR in the INS-1 insulin-secreting cell line is demonstrated here to be triggered by a GLP-1 receptor agonist (exendin-4), by pharmacological agents that stimulate cAMP signalling (forskolin, Sp-cAMPS), and by a methylxanthine known to target Ca^{2+} release channels (caffeine). The source of Ca^{2+} mobilized by CICR resides in a thapsigargin- and ryanodine-sensitive compartment, consistent with the proposed role of RYR-2 as a downstream effector of GLP-1 signal transduction. A possible role for IP_3 receptors as mediators of this response appears unlikely given that CICR was not blocked by the IP_3 receptor antagonist xestospongin C. Instead, available evidence indicates that exendin-4 promotes CICR by sensitizing the RYR Ca^{2+} release mechanism to stimulatory effects of cytosolic Ca^{2+}. Sensitization may result, at least in part, from activation of a novel cAMP signalling mechanism since CICR is shown here to be inhibited by the cAMP antagonist 8-Br-Rp-cAMPS, but is relatively insensitive to the PKA inhibitor H-89. INS-1 cells are also shown to express mRNA coding for cAMP-GEF-II, and overexpression of a dominant negative isoform of cAMP-GEF-II leads to a suppression of forskolin-induced CICR. Taken together, such findings provide evidence in support of an emerging model of Ca^{2+} signalling whereby cAMP-GEFs such as cAMP-GEF-II mediate the cAMP-dependent mobilization of Ca^{2+} from endoplasmic reticulum Ca^{2+} stores.

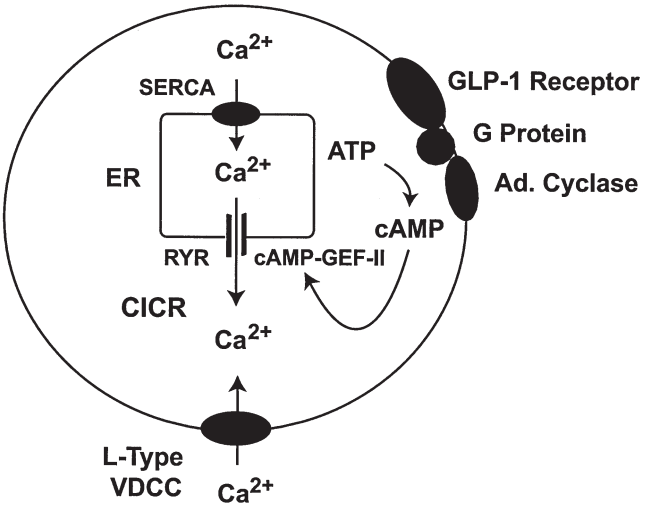

Figure 8. Model illustrating the role of cAMP and cAMP-GEF-II as mediators of CICR

Occupancy of GLP-1 receptors stimulates cAMP production, an effect mediated by G proteins and adenylyl cyclase (Ad. Cyclase). SERCA-mediated Ca^{2+} uptake maintains the filling state of ER Ca^{2+} stores. The action of cAMP is mediated by cAMP-GEF-II. Sensitization of the ER Ca^{2+} release mechanism by cAMP allows RYR-regulated CICR in response to entry of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (VDCCs).

Ca^{2+} release channels responsible for CICR correspond to RYR-2

Multiple competing hypotheses exist concerning the origins of cAMP-dependent CICR in INS-1 cells, as well as in authentic pancreatic β-cells. It was proposed that the relevant Ca^{2+} release channels targeted by cAMP correspond to the IP_3-R (Liu *et al.* 1996), whereas evidence for a major role of RYR-2 was also provided (Islam *et al.* 1992, 1998). It should be noted that both types of ER Ca^{2+} release channels are sensitized by cAMP and PKA under appropriate experimental conditions (Burgess *et al.* 1991; Hajnoczky *et al.* 1993; Nakade *et al.* 1994; Hain *et al.* 1995; Ozawa, 1998; Marx *et al.* 2000). To complicate this picture further, it has been proposed that cAMP-elevating agents such as GLP-1 stimulate an increase of $[Ca^{2+}]_i$ by releasing Ca^{2+} stored in insulin-containing secretory granules (Nakagaki *et al.* 2000).

Although all of these possible mechanisms of Ca^{2+} signalling may play some role in GLP-1 signal transduction, the weight of current evidence favours a dominant role for RYR-2 in support of CICR. This conclusion is advanced on the basis of previous studies of insulin-secreting cells where it was demonstrated that CICR originating from a non-mitochondrial source of Ca^{2+} was blocked by ryanodine, but stimulated by caffeine, thimerosal, and 4-chloro-3-ethylphenol (Islam *et al.* 1992, 1998; Leech *et al.* 1994; Gromada *et al.* 1995; Willmott *et al.* 1995*a*; Chen *et al.* 1996; Gamberucci *et al.* 1999; Holz *et al.* 1999; Maechler *et al.* 1999). The action of caffeine is likely to be specific for ryanodine receptors, and is not secondary to this methylxanthine's inhibitory effect on phosphodiesterase, since the action of caffeine was not blocked by the cAMP antagonist 8-Br-Rp-cAMPS. It should also be noted that expression of RYR-2 was previously confirmed in INS-1 cells by immunoblot analysis (Gamberucci *et al.* 1999).

Despite such convincing evidence, the importance of RYR-2 as a mediator of CICR in β-cells has been debated, particularly from the standpoint of the purported stimulatory action of cyclic ADP-ribose (cADP-ribose) at the ryanodine receptor (Takasawa *et al.* 1998). Studies of permeabilized β-cells demonstrated no effect of ryanodine or agents (caffeine, cADP-ribose) known to interact with RYR-2 (Rutter *et al.* 1994; Willmott *et al.* 1995*b*; Webb *et al.* 1996; Tengholm *et al.* 1998, 2000). Such negative findings are possibly related to the labile nature of CICR when studied in permeabilized islet preparations. In contrast, studies of insulinoma cells demonstrated ryanodine to be an inhibitor of caffeine-induced CICR under conditions in which cells were pretreated with caffeine (Chen *et al.* 1996). This pharmacological

manipulation recapitulates the 'use-dependent' antagonist action of ryanodine because it increases the affinity of RYR-2 for ryanodine by gating the channel from its closed to open, and subsequently blocked state (Bianchi, 1997). In this manner, the sensitivity of CICR to ryanodine is revealed. Using this strategy, we demonstrate that pretreatment with caffeine and ryanodine antagonizes CICR in INS-1 cells.

Conclusion

A role for cAMP in support of CICR might be expected if the filling state of ER Ca^{2+} stores is maintained by PKA-dependent sequestration of Ca^{2+} (Yaekura & Yada, 1998). It has also been proposed that sensitization of β-cell CICR results from PKA-mediated phosphorylation of RYR-2, as is known to occur in cardiac myocytes (Marx *et al.* 2000). We find that stimulators of cAMP signalling evoke CICR, whereas the cAMP antagonist 8-Br-Rp-cAMPS suppresses it. Furthermore, we demonstrate that a high concentration of H-89 produces a generalized suppression of Ca^{2+} signalling. However, when H-89 is tested at a lower concentration, one selective for PKA, we find it to be ineffective. Control experiments using cells transfected with CRE-Luc demonstrate further that the PKA-dependent action of forskolin at the cyclic AMP response element is blocked by a low concentration of H-89. It can be concluded, therefore, that high concentrations of H-89 suppress CICR via non-specific effects, rather than by selective inhibition of PKA. Such findings are reminiscent of the prior study of Bode and co-workers in which it was proposed that GLP-1 mobilizes ER Ca^{2+} stores in a PKA-independent manner (Bode *et al.* 1999). Taken together, our new findings lead us to propose that the cAMP-dependent stimulation of CICR might result from an alternative cAMP signalling mechanism, one involving cAMP-GEF-II (Fig. 8). Such a model is particularly noteworthy in light of the newly recognized role of cAMP-GEFs as mediators of β-cell stimulus–secretion coupling (Ozaki *et al.* 2000; Leech *et al.* 2000). Although it has yet to be ascertained exactly how cAMP-GEF-II exerts its action in this system, previous studies demonstrated that Rap1b, an immediate downstream effector of cAMP-GEF-II, does interact with SERCA (Lacabaratz-Porret *et al.* 1998). Alternatively, cAMP-GEF-II might interact with ion channels such as RYR-2, a possibility suggested by its reported ability to exhibit direct protein–protein interactions with the sulfonlyurea receptor (SUR1), a subunit of cell surface K-ATP channels (Ozaki *et al.* 2000).

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A. & Wollheim, C. B. (1992). Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* **130**, 167–178.

Berridge, M. J. (1996). Microdomains and elemental events in calcium signalling. *Cell Calcium* **20**, 95–96.

Berridge, M. J. (1997). Elementary and global aspects of calcium signalling. *Journal of Experimental Biology* **200**, 315–319.

Berridge, M. J. (1998). Neuronal calcium signaling. *Neuron* **21**, 13–26.

Bianchi, C. P. (1997). Conformation state of the ryanodine receptor and functional effects of ryanodine on skeletal muscle. *Biochemical Pharmacology* **53**, 909–912.

Bode, H. P., Moormann, B., Dabew, R. & Goke, B. (1999). Glucagon-like peptide 1 elevates cytosolic calcium in pancreatic β-cells independently of protein kinase A. *Endocrinology* **140**, 3919–3927.

Bootman, M. D. & Berridge, M. J. (1995). The elemental principles of calcium signaling. *Cell* **83**, 675–678.

Burgess, G. M., Bird, G. S., Obie, J. F. & Putney, J. W. Jr (1991). The mechanism for synergism between phospholipase C- and adenylyl cyclase-linked hormones in liver. Cyclic AMP-dependent kinase augments inositol trisphosphate-mediated Ca^{2+} mobilization without increasing the cellular levels of inositol polyphosphates. *Journal of Biological Chemistry* **266**, 4772–4781.

Buteau, J., Roduit, R., Susini, S. & Prentki, M. (1999). Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase, and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in β (INS-1)-cells. *Diabetologia* **42**, 856–864.

Cheek, T. R., Moreton, R. B., Berridge, M. J., Stauderman, K. A., Murawsky, M. M. & Bootman, M. D. (1993). Quantal Ca^{2+} release from caffeine-sensitive stores in adrenal chromaffin cells. *Journal of Biological Chemistry* **268**, 27076–27083.

Chen, T. H., Lee, B., Yang, C. & Hsu, W. H. (1996). Effects of caffeine on intracellular Ca^{2+} release and Ca^{2+} influx in a clonal β-cell line RINm5F. *Life Sciences* **58**, 983–990.

de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. & Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**, 474–477.

Drucker, D. J. (1998). Glucagon-like peptides. *Diabetes* **47**, 159–169.

Fabiato, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

Frodin, M., Sekine, N., Roche, E., Filloux, C., Prentki, M., Wollheim, C. B. & Van Obberghen, E. (1995). Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting β-cell line, INS-1. *Journal of Biological Chemistry* **270**, 7882–7889.

Gamberucci, A., Fulceri, R., Pralong, W., Banhegyi, G., Marcolongo, P., Watkins, S. L. & Benedetti, A. (1999). Caffeine releases a glucose-primed endoplasmic reticulum Ca^{2+} pool in the insulin secreting cell line INS-1. *FEBS Letters* **446**, 309–312.

Gromada, J., Anker, C., Bokvist, K., Knudsen, L. B. & Wahl, P. (1998*a*). Glucagon-like peptide-1 receptor expression in *Xenopus* oocytes stimulates inositol trisphosphate-dependent intracellular Ca^{2+} mobilization. *FEBS Letters* **425**, 277–280.

Gromada, J., Bokvist, K., Ding, W. G., Holst, J. J., Nielsen, J. H. & Rorsman, P. (1998*b*). Glucagon-like peptide 1 (7–36) amide stimulates exocytosis in human pancreatic β-cells by both proximal and distal regulatory steps in stimulus-secretion coupling. *Diabetes* **47**, 57–65.

Gromada, J., Dissing, S., Bokvist, K., Renstrom, E., Frokjaer-Jensen, J., Wulff, B. S. & Rorsman, P. (1995). Glucagon-like peptide-1 increases cytoplasmic Ca^{2+} in insulin-secreting βTC3-cells by enhancement of intracellular Ca^{2+} mobilization. *Diabetes* **44**, 767–774.

Gromada, J., Holst, J. J. & Rorsman, P. (1998*c*). Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. *Pflügers Archiv* **435**, 583–594.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

Guo, X., Przywara, D. A., Wakade, T. D. & Wakade, A. R. (1996). Exocytosis coupled to mobilization of intracellular calcium by muscarine and caffeine in rat chromaffin cells. *Journal of Neurochemistry* **67**, 155–162.

Hain, J., Onoue, H., Mayrleitner, M., Fleischer, S. & Schindler, H. (1995). Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from cardiac muscle. *Journal of Biological Chemistry* **270**, 2074–2081.

Hajnoczky, G., Gao, E., Nomura, T., Hoek, J. B. & Thomas, A. P. (1993). Multiple mechanisms by which protein kinase A potentiates inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization in permeabilized hepatocytes. *Biochemical Journal* **293**, 413–422.

Hidaka, H. & Kobayashi, R. (1992). Pharmacology of protein kinase inhibitors. *Annual Review of Pharmacology and Toxicology* **32**, 377–397.

Holz, G. G. & Habener, J. F. (1992). Signal transduction crosstalk in the endocrine system: pancreatic β-cells and the glucose competence concept. *Trends in Biochemical Sciences* **17**, 388–393.

Holz, G. G., Kuhtreiber, W. M. & Habener, J. F. (1993). Pancreatic β-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). *Nature* **361**, 362–365.

Holz, G. G., Leech, C. A., Heller, R. S., Castonguay, M. & Habener, J. F. (1999). cAMP-dependent mobilization of intracellular Ca^{2+} stores by activation of ryanodine receptors in pancreatic β-cells. A Ca^{2+} signaling system stimulated by the insulinotropic hormone glucagon-like peptide-1-(7–37). *Journal of Biological Chemistry* **274**, 14147–14156.

Islam, M. S., Leibiger, I., Leibiger, B., Rossi, D., Sorrentino, V., Ekstrom, T. J., Westerblad, H., Andrade, F. H. & Berggren, P. O. (1998). In situ activation of the type 2 ryanodine receptor in pancreatic β-cells requires cAMP-dependent phosphorylation. *Proceedings of the National Academy of Sciences of the USA* **95**, 6145–6150.

Islam, M. S., Rorsman, P. & Berggren, P. O. (1992). Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells. *FEBS Letters* **296**, 287–291.

Kang, G. & Holz, G. G. (2000). Functional coupling of intracellular Ca^{2+} stores to exocytosis of insulin in pancreatic β-cells. *Biophysical Journal* **78**, 146A.

Kang, G. & Holz, G. G. (2001). Ca^{2+}-induced Ca release (CICR) triggers exocytosis and endocytosis in pancreatic β-cells. *Biophysical Journal* **80**, 136A.

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. & Graybiel, A. M. (1998). A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**, 2275–2279.

Kieffer, T. J. & Habener, J. F. (1999). The glucagon-like peptides. *Endocrine Reviews* **20**, 876–913.

Lacabaratz-Porret, C., Corvazier, E., Kovacs, T., Bobe, R. & Bredoux, R. (1998). Platelet sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 3b and Rap1b: interrelation and regulation in physiopathology. *Biochemical Journal* **332**, 413–422.

Leech, C. A., Holz, G. G., Chepurny, O. & Habener, J. F. (2000). Expression of cAMP-regulated guanine nucleotide exchange factors in pancreatic β-cells. *Biochemical and Biophysical Research Communications* **278**, 44–47.

Leech, C. A., Holz, G. G. & Habener, J. F. (1994). Voltage-independent calcium channels mediate slow oscillations of cytosolic calcium that are glucose dependent in pancreatic β-cells. *Endocrinology* **135**, 365–372.

Lester, L. B., Langeberg, L. K. & Scott, J. D. (1997). Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. *Proceedings of the National Academy of Sciences of the USA* **94**, 14942–14947.

Liu, Y. J., Grapengiesser, E., Gylfe, E. & Hellman, B. (1996). Crosstalk between the cAMP and inositol trisphosphate-signalling pathways in pancreatic β-cells. *Archives of Biochemistry and Biophysics* **334**, 295–302.

Maechler, P., Kennedy, E. D., Sebo, E., Valeva, A., Pozzan, T. & Wollheim, C. B. (1999). Secretagogues modulate the calcium concentration in the endoplasmic reticulum of insulin-secreting cells. Studies in aequorin-expressing intact and permeabilized INS-1 cells. *Journal of Biological Chemistry* **274**, 12583–12592.

Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N. & Marks, A. R. (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365–376.

Montrose-Rafizadeh, C., Avdonin, P., Garant, M. J., Rodgers, B. D., Kole, S., Yang, H., Levine, M. A., Schwindinger, W. & Bernier, M. (1999). Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. *Endocrinology* **140**, 1132–1140.

Nabauer, M., Callewaert, G., Cleemann, L. & Morad, M. (1989). Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* **244**, 800–803.

Nakade, S., Rhee, S. K., Hamanaka, H. & Mikoshiba, K. (1994). Cyclic AMP-dependent phosphorylation of an immunoaffinity-purified homotetrameric inositol 1,4,5-trisphosphate receptor (type I) increases Ca^{2+} flux in reconstituted lipid vesicles. *Journal of Biological Chemistry* **269**, 6735–6742.

Nakagaki, I., Sasaki, S., Hori, S. & Kondo, H. (2000). Ca^{2+} and electrolyte mobilization following agonist application to the pancreatic β-cell line HIT. *Pflügers Archiv* **440**, 828–834.

Narita, K., Akita, T., Hachisuka, J., Huang, S., Ochi, K. & Kuba, K. (2000). Functional coupling of Ca^{2+} channels to ryanodine receptors at presynaptic terminals. Amplification of exocytosis and plasticity. *Journal of General Physiology* **115**, 519–532.

Narita, K., Akita, T., Osanai, M., Shirasaki, T., Kijima, H. & Kuba, K. (1998). A Ca^{2+}-induced Ca^{2+} release mechanism involved in asynchronous exocytosis at frog motor nerve terminals. *Journal of General Physiology* **112**, 593–609.

Niggli, E. (1999). Localized intracellular calcium signaling in muscle: calcium sparks and calcium quarks. *Annual Review of Physiology* **61**, 311–335.

Ozaki, N., Shibasaki, T., Kashima, Y., Miki, T., Takahashi, K., Ueno, H., Sunaga, Y., Yano, H., Matsuura, Y., Iwanaga, T., Takai, Y. & Seino, S. (2000). cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nature Cell Biology* **2**, 805–811.

Ozawa, T. (1998). Cyclic AMP induces ryanodine-sensitive Ca^{2+} release from microsomal vesicles of rat parotid acinar cells. *Biochemical and Biophysical Research Communications* **246**, 422–425.

Patterson, G., Day, R. N. & Piston, D. (2001). Fluorescent protein spectra. *Journal of Cell Science* **114**, 837–838.

Rutter, G. A., Theler, J. M., Li, G. & Wollheim, C. B. (1994). Ca^{2+} stores in insulin-secreting cells: lack of effect of cADP ribose. *Cell Calcium* **16**, 71–80.

Skoglund, G., Hussain, M. A. & Holz, G. G. (2000). Glucagon-like peptide 1 stimulates insulin gene promoter activity by protein kinase A-independent activation of the rat insulin I gene cAMP response element. *Diabetes* **49**, 1156–1164.

Susini, S., Van Haasteren, G., Li, S., Prentki, M. & Schlegel, W. (2000). Essentiality of intron control in the induction of c-fos by glucose and glucoincretin peptides in INS-1 β-cells. *FASEB Journal* **14**, 128–136.

Takasawa, S., Akiyama, T., Nata, K., Kuroki, M., Tohgo, A., Noguchi, N., Kobayashi, S., Kato, I., Katada, T. & Okamoto, H. (1998). Cyclic ADP-ribose and inositol 1,4,5-trisphosphate as alternate second messengers for intracellular Ca^{2+} mobilization in normal and diabetic β-cells. *Journal of Biological Chemistry* **273**, 2497–2500.

Tengholm, A., Hagman, C., Gylfe, E. & Hellman, B. (1998). In situ characterization of nonmitochondrial Ca^{2+} stores in individual pancreatic β-cells. *Diabetes* **47**, 1224–1230.

Tengholm, A., Hellman, B. & Gylfe, E. (2000). Mobilization of Ca^{2+} stores in individual pancreatic β-cells permeabilized or not with digitonin or α-toxin. *Cell Calcium* **27**, 43–51.

Thorens, B. (1992). Expression cloning of the pancreatic β-cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proceedings of the National Academy of Sciences of the USA* **89**, 8641–8645.

Tse, A., Tse, F. W., Almers, W. & Hille, B. (1993). Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotropes. *Science* **260**, 82–84.

Tse, F. W., Tse, A., Hille, B., Horstmann, H. & Almers, W. (1997). Local Ca^{2+} release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron* **18**, 121–132.

Usachev, Y. M. & Thayer, S. A. (1999). Controlling the urge for a Ca^{2+} surge: all-or-none Ca^{2+} release in neurons. *Bioessays* **9**, 743–750.

von Ruden, L. & Neher, E. (1993). A Ca^{2+}-dependent early step in the release of catecholamines from adrenal chromaffin cells. *Science* **262**, 1061–1065.

Webb, D. L., Islam, M. S., Efanov, A. M., Brown, G., Kohler, M., Larsson, O. & Berggren, P. O. (1996). Insulin exocytosis and glucose-mediated increase in cytoplasmic free Ca^{2+} concentration in the pancreatic β-cell are independent of cyclic ADP-ribose. *Journal of Biological Chemistry* **271**, 19074–19079.

Wheeler, M. B., Lu, M., Dillon, J. S., Leng, X. H., Chen, C. & Boyd, A. E. (1993). Functional expression of the rat glucagon-like peptide-I receptor, evidence for coupling to both adenylyl cyclase and phospholipase C. *Endocrinology* **133**, 57–62.

Willmott, N. J., Galione, A. & Smith, P. A. (1995*a*). Nitric oxide induces intracellular Ca^{2+} mobilization and increases secretion of incorporated 5-hydroxytryptamine in rat pancreatic β-cells. *FEBS Letters* **371**, 99–104.

Willmott, N. J., Galione, A. & Smith, P. A. (1995*b*). A cADP-ribose antagonist does not inhibit secretagogue-, caffeine- and nitric oxide-induced Ca^{2+} responses in rat pancreatic β-cells. *Cell Calcium* **18**, 411–419.

Yaekura, K. & Yada, T. (1998). $[Ca^{2+}]_i$-reducing action of cAMP in rat pancreatic β-cells: involvement of thapsigargin-sensitive stores. *American Journal of Physiology* **274**, C513–521.

Zawalich, W. S. & Zawalich, K. C. (1996). Glucagon-like peptide-1 stimulates insulin secretion but not phosphoinositide hydrolysis from islets desensitized by prior exposure to high glucose or the muscarinic agonist carbachol. *Metabolism* **45**, 273–278.

Zawalich, W. S., Zawalich, K. C. & Rasmussen, H. (1993). Influence of glucagon-like peptide-1 on β-cell responsiveness. *Regulatory Peptides* **44**, 277–283.

Acknowledgements

G.G.H. acknowledges the support of the National Institutes of Health (R01-DK45817, R01-DK52166) and the American Diabetes Association (Research Grant Award). The authors acknowledge the gift of cAMP-GEF-II expression vectors from Dr Susumu Seino. Excitation spectra for fura-2 and EYFP illustrated in Fig. 1*B* were derived from data supplied by Molecular Probes, Inc. and CLONETECH Lab., Inc. (Patterson *et al.* 2001), respectively.

Corresponding author

G. G. Holz: Medical Sciences Building Rm. 442, 550 First Avenue, New York, NY 10016, USA.

Email: holzg01@popmail.med.nyu.edu