Abscisic Acid Is an Endogenous Stimulator of Insulin Release from Human Pancreatic Islets with Cyclic ADP Ribose as Second Messenger*

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Abscisic acid (ABA) is a plant stress hormone recently identified as an endogenous pro-inflammatory cytokine in human granulocytes. Because paracrine signaling between pancreatic β cells and inflammatory cells is increasingly recognized as a pathogenetic mechanism in the metabolic syndrome and type II diabetes, we investigated the effect of ABA on insulin secretion. Nanomolar ABA increases glucose-stimulated insulin secretion from RIN-m and INS-1 cells and from murine and human pancreatic islets. The signaling cascade triggered by ABA in insulin-releasing cells sequentially involves a pertussis toxin-sensitive G protein, cAMP overproduction, protein kinase A-mediated activation of the ADP-ribosyl cyclase CD38, and cyclic ADP-ribose overproduction. ABA is rapidly produced and released from human islets, RIN-m, and INS-1 cells stimulated with high glucose concentrations. In conclusion, ABA is an endogenous stimulator of insulin secretion in human and murine pancreatic β cells. Autocrine release of ABA by glucose-stimulated pancreatic β cells, and the paracrine production of the hormone by activated granulocytes and monocytes suggest that ABA may be involved in the physiology of insulin release as well as in its dysregulation under conditions of inflammation.

Abscisic acid (ABA)[2] is a plant hormone regulating several important physiological functions related to the response of higher Metaphyta to abiotic stress (temperature variations, water, and nutrient availability) (1). A similar physiological role for ABA has been recently unveiled also in lower Metazoa (marine sponges and Hydroids), where autocrine production of ABA in response to environmental stimuli, temperature rise in sponges or light exposure in Hydroids, has been shown to stimulate water filtration and tissue regeneration, respectively (2–4). The discovery of the synthesis and functional activity of a plant hormone in lower Metazoa suggested a possible role for ABA in animal cells. Indeed, most recent results indicate that ABA production occurs in human granulocytes stimulated with physical (temperature rise, latex beads) or chemical (phorbol myristate acetate) stimuli and that ABA activates several functional activities of granulocytes (including phagocytosis, migration, production of reactive oxygen species, and nitric oxide) involved in their defensive function (5). Thus, ABA behaves as an endogenous pro-inflammatory hormone in human granulocytes.

The remarkable conservation of the role of ABA as a stress signal from plants to humans is paralleled by the strikingly similar signal transduction pathway of the hormone in plants and animals. This pathway sequentially involves phosphorylation and activation of ADP-ribosyl cyclase (ADPRC), overproduction of the universal calcium mobilizer cyclic ADP-ribose (cADPR) (6–8), and increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (2, 4, 9). In human granulocytes, ABA binding to the plasma membrane occurs through a pertussis toxin (PTX)-sensitive receptor-G protein complex, leading to a rapid increase of the $[cAMP]_i$, activation of protein kinase A (PKA), phosphorylation of the ADPRC CD38 with cADPR overproduction, eventually leading to an increase of the $[Ca^{2+}]_i$ (5).

The following considerations are suggested to explore whether ABA had an effect on insulin secretion from mammalian β cells: (i) acute physical stress (trauma, infections, and fever) is known to affect blood glucose levels, inducing hyperglycemia through complex mechanisms, partly involving the action of catecholamines and cortisol but also because of the effects of pro-inflammatory cytokines on pancreatic β cell function (10–12); (ii) the second messengers involved in ABA signaling, cAMP and cADPR, are known to play a role in the signaling pathway leading to glucose-induced insulin secretion (13–17).

[2] The abbreviations used are: ABA, (±)-abscisic acid; cADPR, cyclic ADP-ribose; ADPRC, ADP-ribosyl cyclase; $[Ca^{2+}]_i$, intracellular calcium concentration; PKA, protein kinase A; I-PKA, protein kinase A inhibitor; PTX, pertussis toxin; PCA, perchloric acid; HBSS, Hanks' balanced salt solution; KRH, Krebs-Ringer-Hepes-buffer; LG, low glucose; HG, high glucose; MoAb, monoclonal antibody; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; 8-Br-cAMP, 8-bromo-cAMP; SI, Stimulation Index.

Results obtained indicate that ABA, at nanomolar concentrations, potentiates insulin release in response to glucose in rat insulinoma cells (RIN-m and INS-1) and in human and murine pancreatic islets. The signaling pathway of ABA in insulin-releasing cells involves a PTX-sensitive G protein, cAMP, PKA, and cADPR. High glucose concentrations stimulate ABA production and release from human and rat β cells, indicating that ABA is an endogenous stimulator of insulin release. The fact that ABA is also produced by inflammatory cells, granulocytes (5) and monocytes (18), suggests that this hormone may contribute to the complex network of cytokine signals exchanged between inflammatory cells and pancreatic β cells, which is increasingly recognized as a fundamental mechanism in the development of the metabolic syndrome and diabetes (19–22).

EXPERIMENTAL PROCEDURES

Materials—The [^{3}H]cAMP assay system was purchased from GE Healthcare. The protein kinase A inhibitor (peptide sequence 14–22, myristoylated, I-PKA) was obtained from Calbiochem. Ryanodine was obtained from Ascent Scientific (Weston-super-Mare, UK). Dulbecco's modified Eagle's medium and RPMI 1640 medium were purchased from Cambrex BioScience (Milano, Italy). The IB4 anti-CD38 monoclonal antibody (MoAb) was kindly provided by Prof. F. Malavasi, University of Torino, Italy. ($\pm$)-*cis,trans*-ABA (ABA) and all other chemicals were obtained from Sigma.

Cell Culture—The rat insulinoma RIN-m cell line was obtained from ATCC and cultured, according to the Product Information Sheet, in RPMI 1640 medium with 2 mM glutamine, modified to contain 10 mM Hepes, 1 mM sodium pyruvate, 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate, supplemented with 10% fetal bovine serum. The rat insulinoma cell line INS-1 was kindly provided by Prof. F. Beguinot (Federico II University of Napoli, Italy) and cultured in the same medium as RIN-m cells, supplemented with 50 μM 2-mercaptoethanol. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

Human and Murine Pancreatic Islets—Human pancreatic islets were obtained from S. Raffaele Institute (Milano, Italy) under the framework of the European Consortium for Islet Transplantation, Islets for Research Distribution Program. Briefly, human islets of Langerhans were obtained from the pancreas of heart-beating multiorgan donors as described (23). Islet preparations, which could not be used for transplantation because of low total islet number, were used after approval by the local Ethical Committee. Islets used for the experiments contained $\geq$90% endocrine cells, with $\leq$20% α cells. Blood-derived cells were microscopically undetectable in the islet preparations washed by sedimentation prior to their utilization.

After isolation, human islets (10, 000) were maintained at 37 °C in a humidified atmosphere (5% CO_2), in CMRL-medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate (Euroclone, Celbio), and 2 mM glutamine, and shipped in the same medium. Upon arrival, human pancreatic islets were centrifuged at $200 \times g$ for 2 min, resuspended (200 islets/ml) in an oxygenated Low Glucose buffer (LG-KRH: 129 mM NaCl, 5 mM NaHCO$_3$, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1 mM CaCl$_2$, 1.2 mM MgSO$_4$, 10 mM Hepes, 0.5% bovine serum albumin, 2 mM glucose) and immediately used to evaluate insulin release, cADPR content, and ABA release, as detailed below. Murine pancreatic islets were purified from 8-week-old male C57BL/6Ncrl mice (Charles Rivers SpA, Calco, Italy), as described previously (24).

Insulin Release—RIN-m and INS-1 cells were seeded in a 24-well plate (10^5 cells/well). After 48 h, the culture medium was replaced, and cells were incubated for 4 h in Low Glucose medium (LG-medium: Dulbecco's modified Eagle's medium containing 5.5 mM glucose and supplemented with 2 mM glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin). The medium was discarded, and cells were treated as follows. RIN-m cells were incubated for 15 min or for 1 h at 37 °C with fresh LG-medium or High Glucose medium (HG-medium: same as LG-medium supplemented with glucose to 16.7 mM final concentration) in the presence or absence of different concentrations of ABA; INS-1 cells were incubated for 30 min in LG-KRH or HG-KRH (same composition as LG-buffer, but containing 20 mM glucose) in the presence or absence of different concentrations of ABA. The medium was recovered and centrifuged at $5,000 \times g$ for 1 min, and insulin concentration was determined by EIA (Rat Insulin Enzyme Immunoassay Kit, SpiBio, Montigny le Bretonneux, France).

The human and murine islet suspensions were divided into Eppendorf tubes (100 human or 20 murine islets per determination); after islet sedimentation, supernatants were discarded, and islets were washed once by sedimentation with 0.5 ml of LG-KRH. Islets were incubated in LG-KRH (0.5 and 0.2 ml for human and murine islets, respectively) for 7, 30, or 120 min; supernatants were recovered, centrifuged at $5,000 \times g$ for 1 min, and saved for determination of the insulin content ("basal value"). LG- or HG-KRH, with or without ABA, were then added to the islets for 7, 30, or 120 min; supernatants were recovered, centrifuged at $5,000 \times g$ for 1 min, and saved for determination of the insulin content ("stimulated value"). Insulin concentration was determined with an EIA kit that recognizes rat, murine, and human insulin (rat insulin enzyme immunoassay kit; SpiBio). Results are expressed as Stimulation Index (SI = stimulated value/basal value).

Determination of Intracellular cAMP Levels—RIN-m and INS-1 cells were seeded in 35×10-mm dishes (10^6 cells/dish). After 48 h, the medium was replaced with fresh LG-medium, and cells were incubated for 4 h at 37 °C. The medium was discarded and replaced with Hanks' balanced salt solution (HBSS). ABA (1 μM) was added (or not, control); incubations were stopped after 0, 1, 2, and 5 min by removal of HBSS and addition of 200 μl of ice-cold PCA (0.6 M). When specified, cells were preincubated for 10 min at 37 °C in the presence of the cAMP phosphodiesterase inhibitor Ro-20-1724 (10 μM final concentration). Cell extracts were collected and centrifuged to remove proteins; the cAMP content was measured on the neutralized cell extracts (25) by a radioimmunoassay (GE Healthcare).

The human islet suspension was divided into Eppendorf tubes (100 islets/determination); after islet sedimentation, supernatants were discarded, and islets were washed once by sedimentation with 0.5 ml of LG-KRH. Islets were incubated in

LG-KRH (0.5 ml) for 30 min at 37 °C, and the cAMP phosphodiesterase inhibitor Ro-20-1724 (10 μM final concentration) was added during the last 10 min. The supernatant was discarded, and LG-KRH, with or without 1 μM ABA, was added to the islets for 1, 5, and 15 min. Incubations were stopped by addition of PCA (0.6 M final concentration); the acid extracts were neutralized, and the cAMP content was determined by radioimmunoassay.

Determination of Intracellular cADPR Levels—RIN-m cells were seeded in 35 × 10-mm dishes (10^6 cells/dish). After 48 h, the medium was replaced with fresh LG, and cells were incubated for 4 h at 37 °C. The medium was discarded and replaced with HBSS. ABA (1 μM) was added (or not, control); incubations were stopped after 5 min by removal of HBSS and addition of 300 μl of ice-cold PCA (0.6 M). Cell extracts were collected and centrifuged to remove proteins; the cADPR content was measured on the neutralized perchloric acid cell extracts with a highly sensitive enzymatic cycling assay (25).

The human islet suspension was divided into Eppendorf tubes (100 islets/determination); after islet sedimentation, supernatants were discarded, and islets were washed once by sedimentation with 0.5 ml of LG-KRH. Islets were incubated in LG-KRH (0.5 ml) for 30 min at 37 °C; the supernatant was discarded, and LG- or HG-KRH, with or without ABA (10 nM to 1 μM), was added to the islets for 10 min. The supernatants were then discarded; PCA (300 μl, 0.6 M) was added to the islets; the acid extracts were neutralized, and the cADPR content was determined with the enzymatic cycling assay (25).

Assays of ADP-ribosyl Cyclase Activity—RIN-m cells (5×10^6 cells) or human pancreatic islets (10^3 islets) were incubated in 0.5 ml of KRH at 37 °C with 0.1 mM β-NAD$^+$, in the absence (control) or in the presence of 10 μM ABA. Aliquots (100 μl) were withdrawn at various times, and the enzymatic reactions were stopped by addition of 220 μl of 0.9 M PCA to each aliquot. The cADPR concentration was measured by enzymatic cycling (25). Protein determination in each sample was performed according to Bradford (26).

Western Blot Analysis of CD38—Human islets (500 islets) and RIN-m cells (10^5 cells) were lysed by sonication (30 s at 3 watts on ice) in the presence of protease and phosphatase inhibitors (Sigma; catalog number P8340 and P2850, respectively), and the protein concentration in the lysates was determined (26). Aliquots of the cell lysates (20 μg protein) were loaded on a 12% SDS-PAGE; Western blot analysis and immunoenzymatic detection of CD38 with the anti-CD38 MoAb IB4 were performed according to instructions of the Immobilon Western kit (Millipore Corp., Billerica, MA).

For CD38 immunopurification, lysates from human islets (containing 0.2 mg of protein) were diluted 2-fold in Tris-buffered saline containing 0.25% (w/v) Nonidet P-40 and preincubated with 5 μl of protein A-Sepharose CL-4B (GE Healthcare) for 30 min at 4 °C on a rotating plate. The resin was removed by centrifugation, and the lysates were incubated in the presence of 3 μg of anti-CD38 MoAb IB4 on a rotating plate for 3 h at 4 °C. Twenty μl of protein A-Sepharose CL-4B were then added, and lysates were incubated for 1 h at 4 °C on a rotating plate. Samples were centrifuged and supernatants were removed. The resin was washed three times with 1.5 ml of RIPA buffer (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40). Elution of immune complexes was then obtained by adding 100 μl of a modified Laemmli sample buffer 4× (8% SDS, 0.4 g/ml sucrose, 80 μg/ml bromphenol blue, 62.5 mM Tris-HCl, pH 6.8), at 99 °C for 10 min. Samples were then centrifuged and the supernatants loaded on a 12% SDS-PAGE. The subsequent Western blot analyses and immunoenzymatic detection either with the IB4 anti-CD38 MoAb or with the anti-phosphoserine MoAb (clone PSR-45; Sigma catalog number P3430) were performed according to instructions of the Immobilon Western kit (Millipore Corp.).

Real Time PCR Analyses—Total RNA was extracted from 10^6 RIN-m cells and from 10^3 human islets using the RNeasy micro kit (Qiagen, Milano, Italy) according to the manufacturer's instructions. Quality and quantity of RNA were analyzed using NanoDrop instrumentation (Nanodrop Technologies, Wilmington, DE). Purified total mRNA (1 μg) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). The cDNA was used as template for real time PCR analysis; reactions were performed in a Chromo-4 detection instrument (MJ Research, Waltham, MA). The *Rattus norvegicus* CD38- and human CD38-specific primers (GenBank™ accession numbers NM_013127 and NM_001775, respectively) were designed by using Beacon Designer 2.0 software (Bio-Rad) and purchased from TIB-MOLBIOL (Genova, Italy); their sequences were as follows: 5′-GTGAAGAATC-CTGAGCATCC-3′ (forward) 5′-TGTTAGCATTGTAGT-AGAGACC-3′ (reverse) for rat CD38 and 5′-GGACACGC-TGCTAGGCTACC-3′ (forward) 5′-CATCACATGGACC-ACATCACAGG-3′ (reverse) for human CD38. Each sample was assayed in triplicate in a 20-μl amplification reaction containing 10 ng of cDNA, 400 nM each of sense and antisense primers, and 10 μl of 2× iQ Custom Sybr Green Supermix (Bio-Rad). The amplification program included an initial denaturation at 95 °C for 3 min followed by 40 cycles of two steps, each comprising heating to 95 and to 61 °C, respectively. Fluorescent products were detected at the last step of each cycle. To verify the purity of the products, a melting curve was produced after each run. Values were normalized to β-actin (reference internal gene) mRNA expression. Statistical analysis of the quantitative real time PCR was obtained using the iQ5 Optical System Software version 1.0 (Bio-Rad) based on the $2^{-\Delta\Delta Ct}$ method, which calculated relative changes in gene expression of the target (CD38) normalized to β-actin and relative to a calibrator. Amplification efficiencies of target and reference genes were determined by generating standard curves. Data are presented as means ± S.D. of three different experiments performed in triplicate.

Detection of ABA—Human pancreatic islets (10^3/assay), RIN-m cells, or INS-1 cells (10^6/assay), human peripheral blood mononuclear cells, isolated by Ficoll-Paque gradient centrifugation (10^6/assay), or murine acinar cells (derived from the purification of pancreatic islets) were incubated for 15 min at 37 °C in LG- or HG-KRH. Cell suspensions were centrifuged ($9,000 \times g$ for 1 min), and 4 volumes of distilled methanol was added to each supernatant. Cell pellets were resuspended in 500 μl of deionized water and sonicated at 3 watts for 10 s; aliquots were saved for determination of protein content (26) and 2 ml

of distilled methanol was added to the cell lysates. Trace amounts of [^{3}H]ABA (3×10^3 cpm, *i.e.* 35 fmol) were added as an internal standard to each sample to allow calculation of the extraction yield, and ABA was extracted as described previously (2). ABA content in extracts from cells and supernatants was routinely measured by a sensitive and specific ELISA kit (Agdia, Elkhart, IN), according to the manufacturer's instructions. ABA identification was confirmed by HPLC-coupled mass spectrometry (mass spectrometry and tandem mass spectra), performed on dedicated methanol extracts of cell pellets and supernatants after HPLC purification of ABA (5).

Statistical Analyses—All parameters were tested by paired *t* test or one-way ANOVA followed by Tukey test or Dunnett's method, as appropriate; *p* values <0.05 were considered significant.

RESULTS

Effect of ABA on Insulin Secretion from RIN-m and INS-1 Cells—Preliminarily, we tested the glucose dependence of insulin release in RIN-m and INS-1 cells. In KRH containing 5.5 mM glucose (LG-buffer) or 16.7 mM glucose (HG-buffer), insulin secretion by RIN-m was 1.3 ± 0.1- or 2.2 ± 0.2-fold higher relative to that in glucose-free KRH. In the presence of 30 mM KCl in HG-buffer, inducing maximal secretion, insulin release was 3.4 ± 0.4-fold higher than in glucose-free KRH ($p < 0.01$ by ANOVA, comparing values in glucose-free, LG, HG, and KCl-HG, $n = 3$). In INS-1 cells, insulin secretion in LG-buffer, HG-buffer, or in KCl-HG buffer was 1.5 ± 0.3-, 2.3 ± 0.4-, and 6.6 ± 1.1-fold higher, respectively, relative to that in glucose-free KRH ($p < 0.001$ by ANOVA, comparing values in glucose-free, LG, HG, and KCl-HG, $n = 3$).

In the presence of 5.5 mM glucose in the culture medium (LG-medium), basal insulin secretion from RIN-m cells (2.6 ± 1.3 ng insulin/10^6 cells/h, $n = 8$) was increased by ABA ~1.6-fold relative to release in LG, with 10 nM of the hormone being as effective as 10 μM (Fig. 1*A*, *gray bars*, $p < 0.001$ by ANOVA, comparing insulin secretion induced by $0–10^4$ nM ABA in LG. No significant difference was found between insulin release in 10, 10^2, 10^3, and 10^4 nM ABA in LG, $p > 0.6$, by Tukey test.). At 1 nM, ABA did not significantly stimulate insulin release from RIN-m cells neither in LG-medium (Fig. 1*A*, *gray bar*) nor in HG-medium (Fig. 1*A*, *black bar*). Insulin secretion (relative to LG) induced by 10 nM ABA in LG was similar ($p = 0.49$ by *t* test, $n = 6$) to that observed in a high glucose, ABA-free medium (HG-medium, 16.7 mM glucose) (Fig. 1*A*, *gray bar* at 10 nM ABA compared with *black bar* at 0 nM ABA).

ABA also potentiated insulin secretion at high glucose concentrations, in a statistically significant manner for ABA concentrations ranging between 10 nM and 10 μM ($p < 0.001$ by ANOVA, $n = 6$) (Fig. 1*A*, *black bars*). No significant difference was observed between insulin release in 10, 10^2, 10^3. and 10^4 nM ABA in HG ($p > 0.06$ by Tukey test).Finally, at each ABA concentration tested, insulin release in HG was significantly higher than in LG (Fig. 1*A*, *black bar* compared with the *gray bar* at the same ABA concentration ($p < 0.001$ by *t* test)).

Also in INS-1 cells, 10 nM ABA increased insulin secretion ~2-fold in LG-buffer as well as in HG-buffer (relative to release in LG without ABA, Fig. 1*B, gray bars*, or to release in HG

without ABA, Fig. 1*B, black bars*). Similarly to what was observed in RIN-m, 10 nM of the hormone was as effective as 10 μM (Fig. 1*B, gray bars*, $p < 0.001$ by ANOVA, comparing insulin secretion induced by $0–10^4$ nM ABA in LG. No significant difference was found between insulin release at 10, 10^2, 10^3, and 10^4 nM ABA in LG, $p > 0.6$ by Tukey test.). At 1 nM, ABA slightly but significantly stimulated insulin release from INS-1 cells in LG-buffer (1.5-fold relative to LG without ABA, Fig. 1*B, gray bar*), as well as in HG-buffer without ABA (1.6-fold Fig. 1*B, black bar*). ABA at 0.1 nM was without effect at both glucose concentrations (Fig. 1*B*).

We investigated whether ABA also increased insulin secretion in the absence of glucose. In RIN-m cells incubated in glucose-free KRH, 1 μM ABA induced insulin secretion (Fig. 1*C, white bars*) and also potentiated insulin secretion in LG-buffer (Fig. 1*C, gray bars*).

Both ABA enantiomers, (+)- and (−)-*cis,trans*-ABA, were similarly effective in stimulating insulin release from RIN-m (Fig. 1*D*), in line with what was observed on the $[Ca^{2+}]_i$ increase in human granulocytes (5). The same inhibitors that were shown to target the ABA signaling pathway in granulocytes (5) were tested for their ability to prevent ABA-induced insulin secretion from RIN-m cells incubated in LG-medium. Pretreatment with PTX abrogated ABA-induced insulin release (Fig. 1*E*). The PKA-specific inhibitor, myristoylated peptide (I-PKA), also abrogated ABA-induced insulin release; conversely, the membrane-permeant cAMP analog, 8-Br-cAMP, an activator of PKA, stimulated insulin secretion (Fig. 1*E*). The ADP-ribosyl cyclase inhibitor nicotinamide and the cADPR antagonists 8-Br-cADPR and ryanodine severely reduced ABA-triggered insulin release (by 85, 70, and 93%, respectively). The causal role of an intracellular calcium increase in the ABA-induced insulin release was confirmed by the almost complete inhibition of insulin release in the presence of the intracellular calcium chelator EGTA-AM (Fig. 1*E*). Altogether, these results indicate involvement of a PTX-sensitive G-protein-receptor complex, cAMP, PKA, and cADPR in the signaling pathway triggered by ABA and leading to insulin release in RIN-m cells. The same signal transduction pathway was shown to mediate ABA-induced functional activation of human granulocytes (5).

Effect of ABA on Insulin Secretion from Murine and Human Pancreatic Islets—Results obtained on the rat insulinoma cell lines prompted us to explore the effect of ABA on murine pancreatic islets. Incubation with ABA for 30 min stimulated insulin release from freshly isolated murine pancreatic islets in a concentration-dependent manner; as low as 1 nM of the hormone significantly increased insulin release in LG, and 10 nM was as effective as 1 μM of the hormone, increasing the SI ~1.6-fold over values in LG (Fig. 2*A*). Maximal stimulation of insulin release, as afforded by HG-KCl, induced a 3.8-fold increase of the SI (Fig. 2*A*). Next, we compared the time course of ABA- and glucose-stimulated insulin release in murine pancreatic islets; glucose induced a faster response than ABA (SI values were 1.51 ± 0.15 *versus* 0.95 ± 0.17 at 7 min and 2.02 ± 0.18 *versus* 1.55 ± 0.012 at 30 min, for HG and 10 nM ABA, respectively), but at 120 min SI values for HG and ABA were not significantly different (2.41 ± 0.19 *versus* 2.32 ± 0.22, $p = 0.6$ by *t* test, $n = 3$) (Fig. 2*B*).
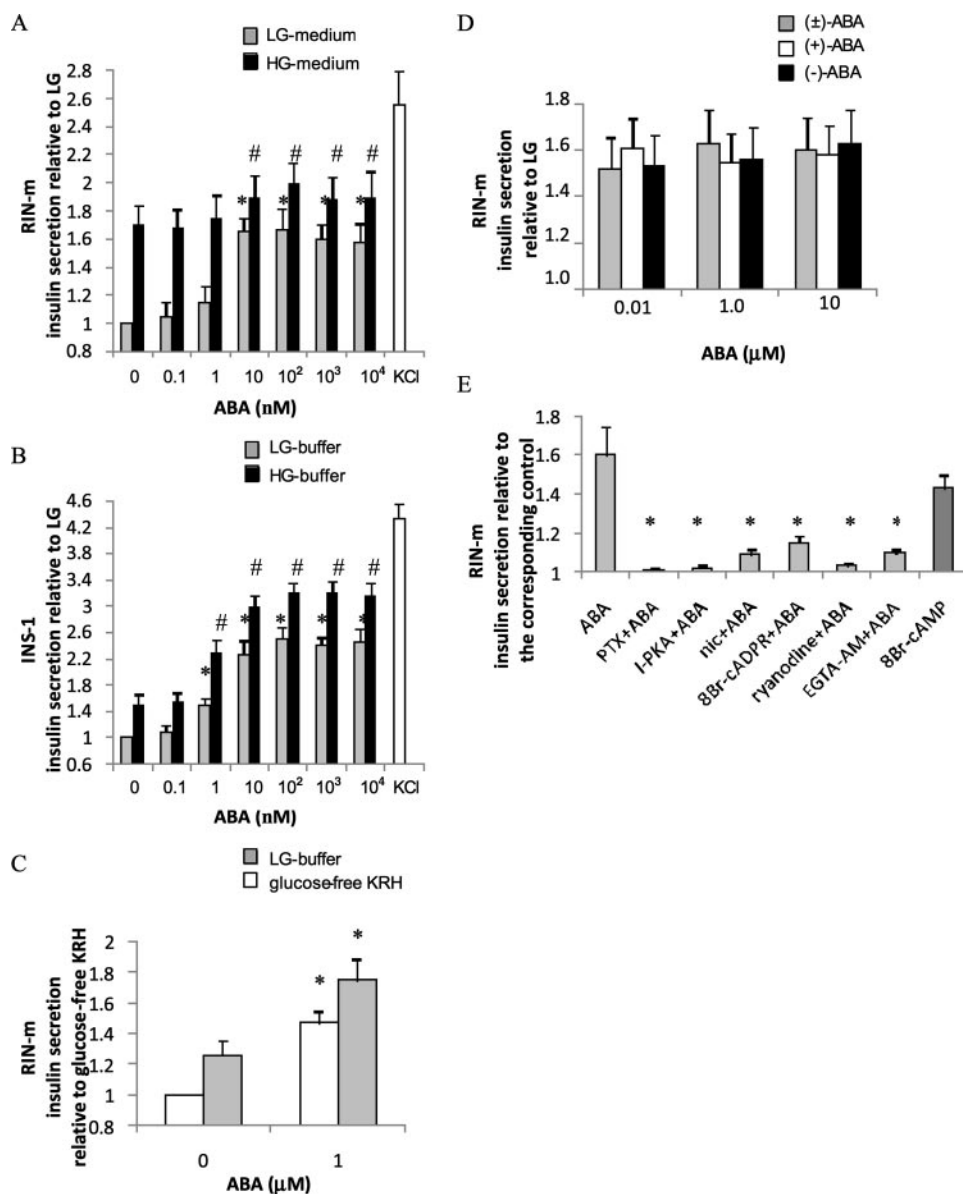
FIGURE 1. **ABA-induced insulin release from RIN-m and INS-1 cells.** *A,* RIN-m cells were incubated for 1 h at 37 °C in LG- or HG-medium, in the absence (0) or in the presence of increasing ABA concentrations, or in the presence of 30 mM KCl in HG-medium (KCl); control, cells in LG without ABA. *, $p < 0.05$ by Tukey test, compared with LG, without ABA; #, $p < 0.05$ by Tukey test, compared with HG without ABA. *B,* INS-1 were incubated for 30 min at 37 °C in LG- or HG-buffer, without (0) or with increasing ABA concentrations, or in the presence of 30 mM KCl in HG-buffer (KCl); control, cells in LG without ABA. *, $p < 0.05$ by Tukey test, compared with LG, without ABA; #, $p < 0.05$ by Tukey test, compared with HG without ABA. *C,* RIN-m were incubated for 1 h at 37 °C in glucose-free KRH or in LG-buffer in the presence or absence of 1 μM ABA; control, cells in glucose-free KRH, without ABA. *, $p < 0.01$ by paired *t* test, compared with the same medium, without ABA. *D,* RIN-m in LG-medium, with the indicated concentrations of (±)-ABA, (+)-ABA, or (−)-ABA; control, cells in LG, without ABA. No significant difference between these bars. *E,* RIN-m were preincubated (or not, ABA) at 37 °C in LG-medium with PTX (2 μg/ml, 1 h), I-PKA (1 μM, 1 h), nicotinamide (20 mM, 5 min), 8-Br-cADPR (50 μM, 20 min), ryanodine (50 μM, 20 min), or EGTA-AM (0.5 mM, 20 min) and then exposed or not to 1 μM ABA for 15 min; for each condition, the control was insulin release from cells in LG, without ABA, in the presence of the indicated compound. At the concentrations and for the times explored, none of the compounds significantly reduced insulin release induced by LG, without ABA ($p > 0.5$ by ANOVA). *, $p < 0.05$ by Dunnett's method, compared with ABA. In parallel, cells were stimulated in LG-medium for 30 min without (control) or with 0.5 mM 8-Br-cAMP. Results are expressed as insulin secretion over control and are the mean ± S.D. of the following number of experiments: *A, n = 6; B, n = 4; C–E, n = 3.*

Stimulation by ABA of insulin release from the rat insulinoma cell lines and from murine pancreatic islets prompted us to explore the effect of the hormone on human pancreatic β cells. At both time points explored, 30 and 120 min from the addition of 10 nM ABA, insulin secretion was increased over values recorded in LG-KRH (Fig. 2*C*). The effect of ABA was already maximal at 10 nM, as higher concentrations (up to 10 μM) did not further increase insulin release (not shown). The SI at 120 min was similar in cells incubated with 10 nM ABA or with HG (SI values were 2.7 and 2.9, respectively, $p = 0.46$ by *t* test; Fig. 2*C*, *black bars*). Stimulation of insulin secretion by HG, however, was faster compared with that exerted by ABA; SI values were 2.3 and 1.5, respectively, at 30 min of incubation, $p < 0.01$ (Fig. 2*C*, *white bars*), similarly to what observed in murine islets. The ABA-induced insulin secretion (measured in LG-KRH) was abrogated or significantly reduced (by ~60%) by pretreatment of human islets with PTX or with 8-Br-cADPR, respectively (Fig. 2*C*), as already observed on RIN-m cells (Fig. 1*E*). Finally, 8-Br-cAMP stimulated insulin secretion, as already reported (27), with an SI remarkably higher at 2 h than at 30 min, similar to what observed with ABA (Fig. 2*C*). These results indicate that human and murine islets respond to nanomolar ABA with an insulin secretion quantitatively similar to that triggered by HG and that a PTX-sensitive G protein and cADPR are involved in the human ABA signal transduction pathway.

ABA Signal Transduction in RIN-m Cells and Human Islets—Inhibition of ABA-induced insulin secretion from RIN-m cells by inhibitors of the ADPRC/cADPR system (nicotinamide, ryanodine, and 8-Br-cADPR) and by the PKA-specific inhibitor (Fig. 1*E*) suggested involvement of the second messengers cADPR and cAMP in ABA signaling. Thus, the intracellular concentrations of cAMP ($[cAMP]_i$) and of cADPR ($[cADPR]_i$) were measured in RIN-m cells following exposure to 1 μM ABA. The $[cAMP]_i$ was explored both in the absence (Fig. 3*A*, *circles*) and in the presence (Fig. 3*A*, *squares*) of the phosphodiesterase inhibitor Ro-20-1724. The basal $[cAMP]_i$ was much higher in RIN-m pretreated with the phosphodiesterase inhibitor compared with untreated cells, as reported also in MIN6 (28) and in INS-1 cells (29); however, the kinetics of $[cAMP]_i$ increase upon addition of ABA were
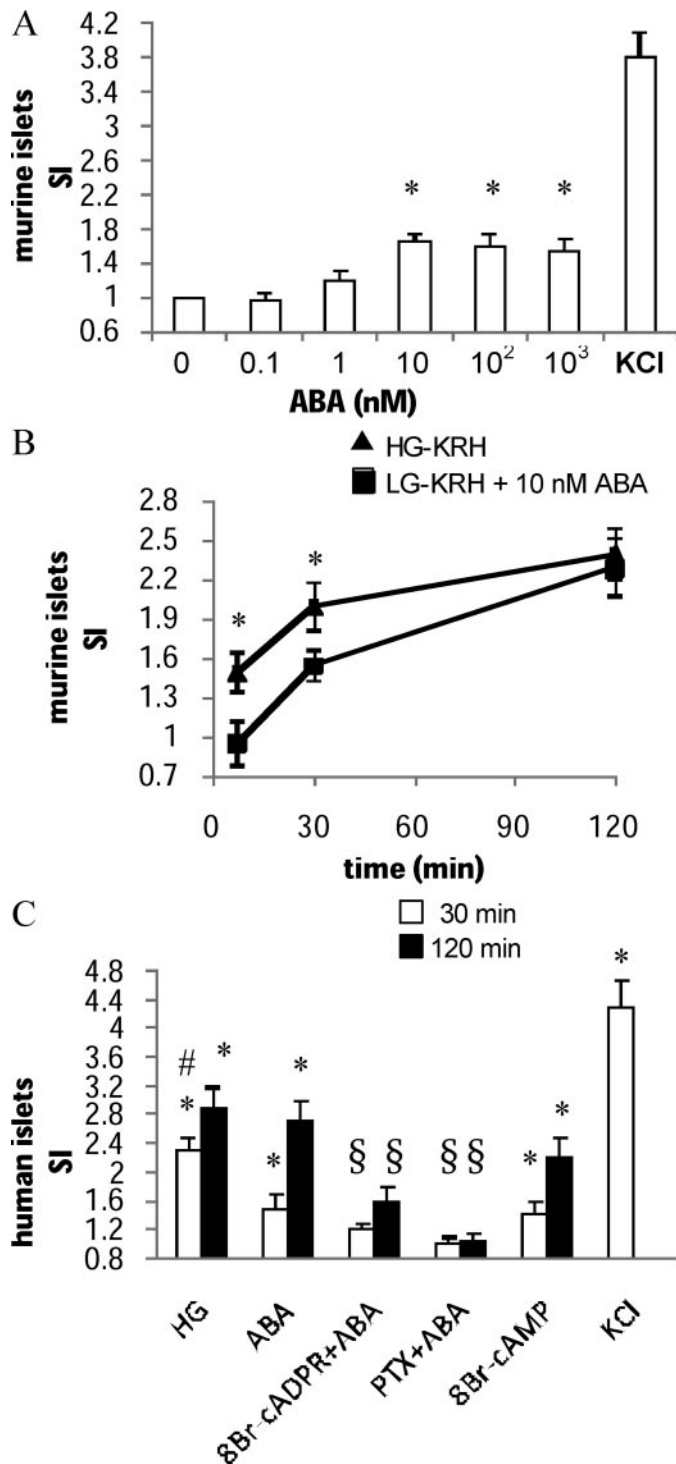
FIGURE 2. **ABA-induced insulin release from murine and human pancreatic islets.** *A*, murine islets (20/assay) were incubated at 37 °C for 30 min in LG-KRH (basal value) and then further incubated for the same time without (0) or with increasing ABA concentrations or in the presence of 30 mM KCl in HG-KRH (KCl) (stimulated value). *, $p < 0.001$ by ANOVA, comparing insulin secretion induced by 0–10^3 nM ABA. No significant difference was found between insulin release in 10, 10^2, and 10^3 nM ABA ($p > 0.6$ by Tukey test). *B*, murine islets (20/assay) were incubated at 37 °C in LG-KRH for 7, 30, or 120 min (basal value) and then further incubated for the same times in LG-KRH, or in HG-KRH, or in LG-KRH containing 10 nM ABA (stimulated value). *, $p < 0.05$ by *t* test, comparing the SI value in HG-KRH to that obtained with ABA in LG-KRH, at the same time point. *C*, human islets (100/assay) were incubated at 37 °C for 30 or 120 min in LG-KRH and then further incubated for the same times in LG-KRH, or in HG-KRH, or in HG-KRH containing 30 mM KCl (KCl), or in LG-KRH containing 10 nM ABA or 0.5 mM 8-Br-cAMP, (stimulated value). *, $p <$

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similar in both experimental conditions, with a significant increase of the $[cAMP]_i$ being recorded after 1 min (Fig. 3*A*). Preincubation of RIN-m cells with PTX, in the presence of the phosphodiesterase inhibitor, did not significantly modify the basal $[cAMP]_i$, as reported in Ref. 30. Conversely, pretreatment with PTX abolished the ABA-induced $[cAMP]_i$ increase (Fig. 3*A*).

Incubation with 1 μM ABA increased the $[cAMP]_i$ also in INS-1 cells, from a time 0 value of 7.5 ± 0.9 to 10.6 ± 1.2 pmol/10^6 cells after 1 min, in the presence of Ro-20-1724 ($p < 0.01$ by *t* test, $n = 4$). 10 nM ABA was as effective as 1 μM of the hormone, whereas 1 nM ABA increased the $[cAMP]_i$ to a slightly lesser extent (1.3 ± 0.1-fold increase relative to time 0) after 1 min.

The $[cADPR]_i$ in RIN-m cells increased upon addition of ABA, with the maximal increase (2.5-fold over basal values in LG) being recorded at 5 min (Fig. 3*B*). 8-Br-cAMP also induced an increase of the $[cADPR]_i$ (3.5-fold over LG values at 5 min), indicating that activation of PKA leads to overproduction of cADPR in RIN-m cells, as already reported in other cells types (31–34, 5). Next, we measured the effect of ABA on the ADPRC activity responsible for cADPR generation. Levels of ADPRC activity were measured on intact cells, using β-NAD$^+$ as substrate. ADPRC activity increased from 0.013 ± 0.002 pmol of cADPR/min/mg of protein (in control RIN-m cells) to 0.031 ± 0.004 pmol cADPR/min/mg protein after 5 min of incubation of RIN-m cells with 10 μM ABA ($p < 0.01$ by *t* test, $n = 3$).

Similarly to what observed in RIN-m, ABA induced an increase of the $[cAMP]_i$ and of the $[cADPR]_i$ also in human islets. The $[cAMP]_i$ was explored in the presence of the phosphodiesterase inhibitor Ro-20-1724; the basal $[cAMP]_i$ was increased upon addition of ABA, with maximal values being recorded after 5 min (Fig. 3*C*). Pretreatment with PTX abolished the ABA-induced $[cAMP]_i$ increase also in human islets (Fig. 3*C*). Maximal values of $[cADPR]_i$ (~2-fold increase over LG) were recorded after 10 min of incubation with ABA and were similar for ABA concentrations ranging between 10 nM and 1 μM, as well as for $(+)$- and $(-)$-ABA (Fig. 3*D*). Preincubation of human islets with PTX for 1 h abrogated the ABA-induced $[cADPR]_i$ increase (Fig. 3*D*), confirming a role for a PTX-sensitive G protein in ABA-triggered human insulin secretion (Fig. 2*C*). The marked 8-Br-cAMP-stimulated cADPR overproduction (Fig. 3*D*) demonstrates that PKA activation leads to an increase of the $[cADPR]_i$ in human islets. Finally, the $[cADPR]_i$ increased 1.5-fold in human islets incubated for 10 min in HG compared with LG (Fig. 3*D*), in agreement with what was previously observed in rat islets (35) and

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0.05 by *t* test, each compared with LG, without ABA. #, $p < 0.01$ by *t* test, compared with 10 nM ABA in LG-KRH. In parallel, human islets were preincubated at 37 °C for 30 or 120 min in LG-KRH in the presence of 8-Br-cADPR (50 μM) or PTX (2 μg/ml) (basal value) and then further incubated for the same times in LG-KRH without or with of 10 nM ABA. At the concentrations and for the times explored, neither 8-Br-cADPR nor PTX significantly reduced insulin release induced by LG, without ABA ($p > 0.5$ by ANOVA). §, $p < 0.05$ by Tukey test, compared with ABA, at the corresponding time. Results are expressed as Stimulation Index (*SI*) and are the mean $\pm$ S.D. of three experiments, each performed with one batch of islets, obtained from 25 mice (*A* and *B*) or from a single human donor (*C*).
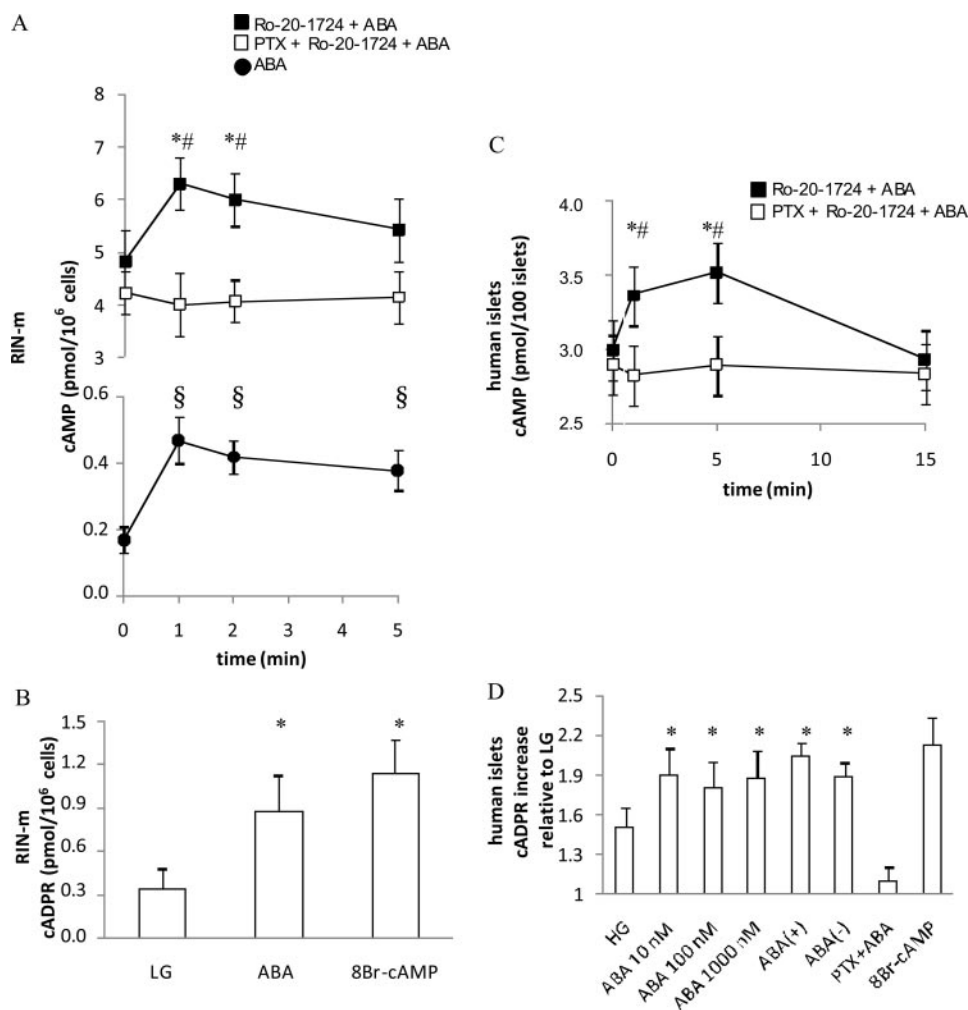
FIGURE 3. **ABA-induced [cAMP]$_i$ and [cADPR]$_i$ increase in RIN-m cells and in human islets.** *A,* ABA (1 μM) was added to RIN-m preincubated or not in the presence of the phosphodiesterase inhibitor Ro-20-1724 for 10 min; [cAMP]$_i$ levels were determined at the indicated times after addition of ABA in extracts of cells untreated or pretreated with PTX (2 μg/ml for 1 h). *, $p < 0.05$ by t test, compared with the time 0 value; §, $p < 0.01$ by t test, compared with the time 0 value; #, $p < 0.01$ by t test, compared with the PTX-pretreated sample, at the same time point. *B,* RIN-m were incubated for 5 min at 37 °C in LG-medium without (*LG*) or with 1 μM ABA or 0.5 mM 8-Br-cAMP, and the [cADPR]$_i$ was determined. *, $p < 0.001$ by t test, compared with LG. *C,* human islets were preincubated with Ro-20-1724 for 10 min; after addition of 1 μM ABA, [cAMP]$_i$ levels were determined at the times indicated in extracts of cells untreated or pretreated with PTX (2 μg/ml for 1 h). *, $p < 0.05$; #, $p < 0.05$ by t test, compared with time 0 and to the PTX-pretreated sample at the same time point, respectively. *D,* human islets were incubated for 10 min at 37 °C in the presence of HG- or LG-KRH without (control) or with ($\pm$)-ABA at the concentrations indicated, or with 100 nM (+)- or ($-$)-ABA, or with 0.5 mM 8-Br-cAMP. In parallel, islets were preincubated in LG-KRH with PTX (2 μg/ml for 1 h) before addition of 100 nM ABA. Results are expressed as cADPR increase relative to controls in LG, without ABA. Basal [cADPR]$_i$ in human islets was 2.0 $\pm$ 0.6 pmol cADPR/10^3 islets ($n = 3$). *, $p < 0.001$ by ANOVA, comparing the cADPR increase induced by 0–10^3 nM ABA and 10^2 nM (+)- or ($-$)-ABA in LG. No significant difference was observed between the cADPR increase at 10, 10^2, and 10 and 10^2 nM (+)- or ($-$)-ABA in LG ($p > 0.7$ by Tukey test). Results are the mean $\pm$ S.D. of the following number of experiments: *A, C,* and *D, n = 3; B, n = 8.*

with the proposed role for cADPR in glucose-stimulated insulin secretion (16).

Similarly to the effect on RIN-m cells, exposure to 10 μM ABA stimulated ADPRC activity in human islets, from a basal value of 0.08 $\pm$ 0.02 pmol of cADPR/min/mg protein to 0.15 $\pm$ 0.03 and 0.11 $\pm$ 0.02 pmol of cADPR/min/mg protein after 5 and 30 min of incubation, respectively ($p < 0.05$ by t test, $n = 4$).

Expression of CD38 in RIN-m cells and in human islets was confirmed by Western blot analysis of cell lysates with an anti-CD38 MoAb, which recognizes both human and rat CD38 (36).

The apparent M_r of rat CD38 was slightly higher compared with the human protein (48 *versus* 44 kDa, respectively), as already observed (37), and expression of CD38 was higher in human islets compared with insulinoma cells (Fig. 4*A*). The different expression level of CD38 in RIN-m and in β cells was confirmed by reverse transcription-PCR (Fig. 4*B*).

As PKA activation by 8-Br-cAMP induced an increase of the [cADPR]$_i$ in human islets (Fig. 3*D*), the possible phosphorylation of CD38 following exposure of islets to ABA was investigated. Indeed, incubation of islets with 10 μM ABA increased the phosphorylation state of CD38 by 2.4- and 2.0-fold over time 0 values after 5 and 30 min, respectively (Fig. 4*C*).

ABA Release from Glucose-stimulated Human Islets and Rat Insulinoma Cell Lines—Results shown in Figs. 1 and 2 indicate that rat and human pancreatic β cells respond to ABA with a glucose-independent insulin secretion and with a potentiation of glucose-induced insulin release. To investigate whether ABA could be produced autocrinally by pancreatic β cells in response to glucose, human islets were incubated in the presence of LG- or HG-KRH and the ABA content in both cell extracts and supernatants was determined by ELISA. The intracellular ABA concentration doubled in islets incubated for 15 min in HG- compared with LG-KRH and a 25-fold increase of ABA content in the islet supernatant was observed (Fig. 5*A*). Similar results were obtained on RIN-m cells; the intracellular ABA concentration doubled in cells exposed to HG for

15 min, and ABA content in HG-medium increased 20-fold over LG values (Fig. 5*B*). Finally, HG also induced ABA release from INS-1 cells, with a 10-fold increase of the ABA content in the medium compared with values measured in LG after 15 min of incubation (Fig. 5*C*). The fact that both rat insulinoma cell lines release ABA when challenged with HG, similarly to human islets, strongly suggests that ABA release can be attributed to pancreatic β cells. Moreover, neither human blood-derived mononuclear cells nor murine exocrine pancreatic cell preparations release ABA when incubated in HG (data not shown), further supporting the conclusion that glucose-in-
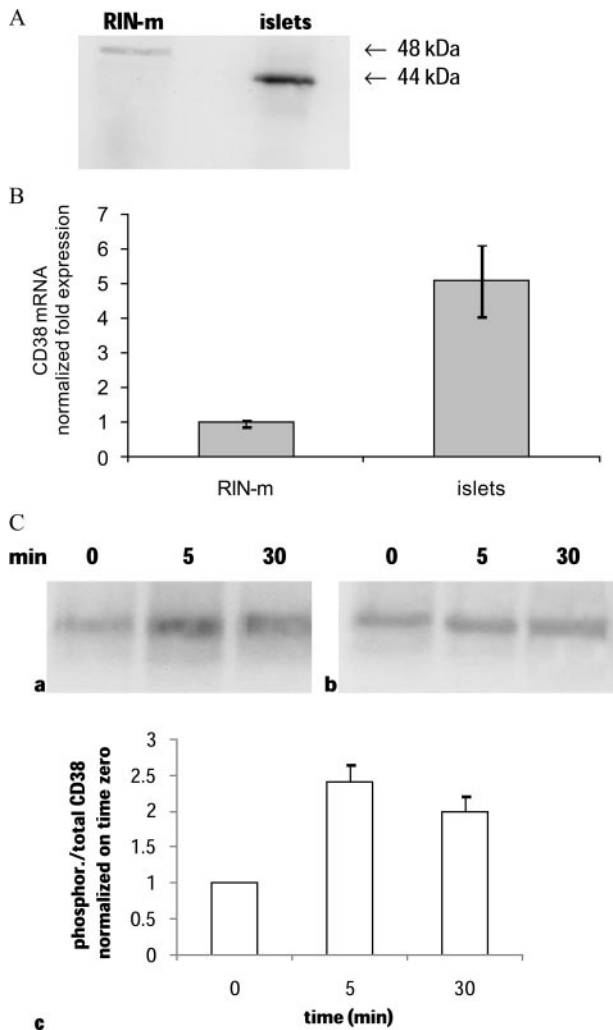
FIGURE 4. **Expression of CD38 in RIN-m cells and in human pancreatic islets.** *A,* Western blot analysis of CD38 in RIN-m cells and in human pancreatic islets; RIN-m and islets were lysed, and 20 μg of proteins were subjected to SDS-PAGE (12%) and Western blot. Detection of CD38 was obtained with an anti-CD38 MoAb (IB4). Results are from a representative experiment (*n* = 3). *B,* real time PCR of CD38 in human islets relative to CD38 in RIN-m, each normalized to β-actin (*n* = 3). *C,* CD38 was immunopurified as described under "Experimental Procedures," from control and ABA-treated (for 5 and 30 min with 10 μM ABA) human islets. Samples were run in duplicate; Western blots were stained with an anti-phosphoserine monoclonal antibody (*panel a*) or with the anti-CD38 MoAb IB4 (*panel b*). Results are from one of three different experiments, yielding comparable results. Quantification of CD38 phosphorylation (*panel c*); ratio of phosphorylated to total CD38, normalized on the ratio at time = 0 (mean ± S.D. from three different experiments).

FIGURE 5. **ABA release from human pancreatic islets, RIN-m, and INS-1 cells stimulated with glucose.** Islets (10^3/determination, 0.5 ml of total volume, *A*), RIN-m (10^6/determination, *B*) or INS-1 (10^6/determination, *C*) were incubated in 0.5 ml (islets) or 1.2 ml (RIN-m and INS-1) of LG- or HG-KRH for 15 min at 20 °C. The ABA content in cells and supernatants was determined by ELISA. Results are expressed as pmol of ABA detected in the cells (*white bars*), or in the supernatants (*gray bars*), or as the sum of the ABA content in cells and supernatants (*black bars*), normalized to 1 mg of cell protein content in the incubation. Results shown are the mean ± S.D. from three experiments (human pancreatic islets were from three different subjects). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by paired *t* test, compared with the corresponding value in LG.

duced ABA release from human islets is not because of contaminating cell types present in the human islet preparation. The fact that glucose stimulates pancreatic cells to release ABA, which in turn increases insulin secretion (Fig. 2*C*), may generate a positive feedback mechanism prolonging and enhancing insulin release in response to glucose.

DISCUSSION

The present results demonstrate that ABA stimulates insulin secretion by pancreatic β cells. The fact that ABA potentiates glucose-induced insulin release (Figs. 1 and 2) and stimulates glucose-independent insulin secretion (Fig. 1) in both rat insulinoma cells (RIN-m) and human pancreatic islets argues
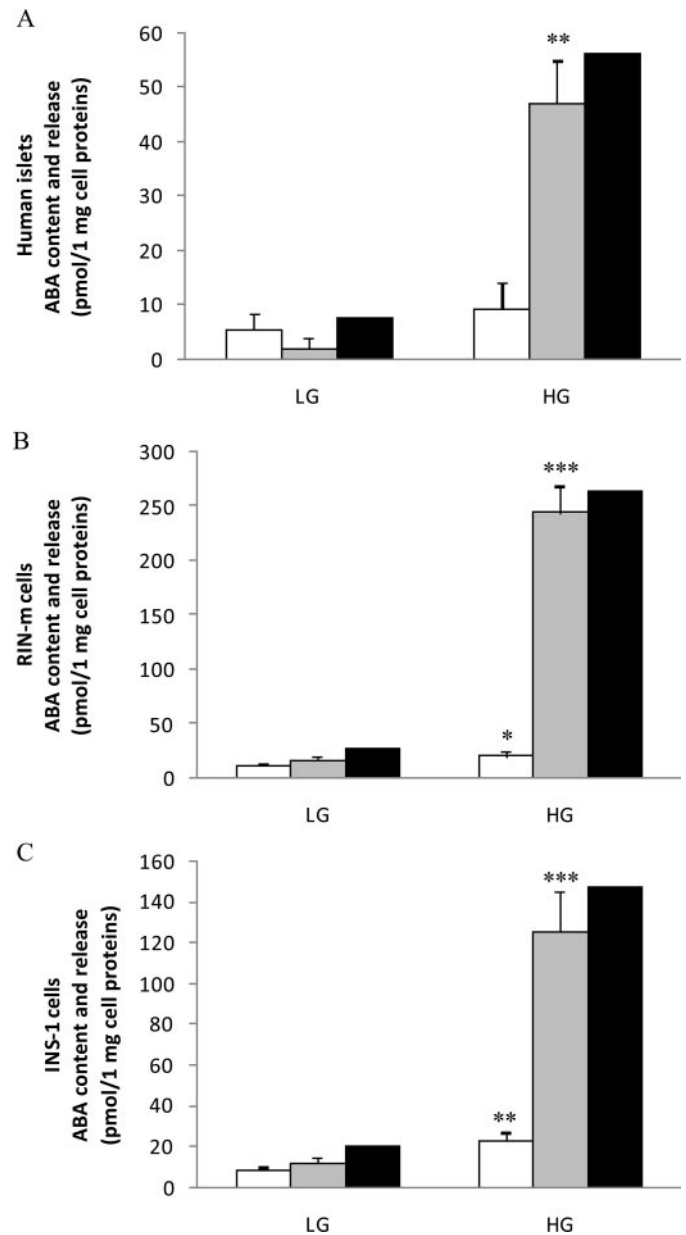
against the possibility that the effect of ABA is mediated by accessory cells (α cells, inflammatory cells, or exocrine cells contaminating the islet preparation). ABA acts on RIN-m cells and on human β cells through the same signaling pathway, sequentially involving a PTX-sensitive G protein (Figs. 1*E* and 3), cAMP overproduction (Fig. 3, *A* and *C*), PKA-mediated CD38 phosphorylation (Fig. 4*B*), and activation and increase of the $[cADPR]_i$ (Fig. 3, *B* and *D*). The same signaling cascade is activated by ABA in human granulocytes (5).

Pretreatment of RIN-m cells with the intracellular Ca^{2+} chelator EGTA-AM prevented the ABA-induced insulin release (Fig. 1*E*). Intracellular Ca^{2+} chelation *per se* does not inhibit the insulin exocytotic pathway, as hypotonic stress-induced insulin release can occur in the presence of intracellular Ca^{2+} chelators (38). Thus, abrogation by EGTA-AM of ABA-induced insulin release indicates that the effect of ABA on insulin secretion is specifically dependent on the increase of the $[Ca^{2+}]_i$, similar to the ABA-triggered stimulation of functional activities in granulocytes (5). The intracellular Ca^{2+} mobilizer cADPR (6, 7) is primarily responsible for the ABA-triggered insulin secretion, as the cADPR antagonists 8-Br-cADPR and ryanodine strongly inhibited (by 80%) the ABA-induced insulin release in RIN-m cells (Fig. 1*E*). 8-Br-cADPR also significantly inhibited (by 70%) the ABA-triggered insulin release from human islets (Fig. 2*C*). Identification of a primary role of cADPR in the ABA-induced insulin release opens the way to an in-depth investigation into the synergism between the ABA- and glucose-stimulated Ca^{2+} signaling pathways, suggested by the synergistic effect of ABA and glucose on insulin release (Fig. 1).

The fact that 8-Br-cADPR and ryanodine do not completely inhibit the ABA-induced insulin release in RIN-m cells (Fig. 1*E*) may suggest involvement of mechanisms of intracellular Ca^{2+} increase other than those related to cADPR and accounting for ≤20% of the ABA-triggered insulin release. No significant increase of the [inositol 1,4,5-trisphosphate]$_i$ was detected in RIN-m cells at the time points (10 and 30 s and 1, 2, and 5 min) investigated following addition of ABA (data not shown). $NAADP^+$, the most potent intracellular Ca^{2+} mobilizer (39), was recently shown to be involved in glucose-stimulated $[Ca^{2+}]_i$ rise in the pancreatic β cell line MIN6 cells (40). As changes in the $[NAADP^+]_i$ following ABA stimulation of RIN-m and human islets were not investigated, a limited role for $NAADP^+$ in the ABA-induced insulin release cannot be ruled out.

In any case, the almost complete inhibition (by >90%, Fig. 1*E*) afforded by I-PKA and ryanodine on the ABA-induced insulin release in RIN-m cells unambiguously indicates a primary role for the cAMP/PKA/cADPR pathway in the ABA signaling cascade. To our knowledge, ABA is the first example of a hormone sequentially activating both second messengers, cAMP and cADPR, individually reported to be involved in insulin release from pancreatic cells (13–17). This fact may account for the observed potentiation of glucose-induced insulin release by ABA.

Regarding the physiological role of ABA in insulin secretion, ABA release from both human islets and rat insulinoma cells in response to HG (Fig. 5) may trigger a positive feedback mechanism, further stimulating insulin secretion. The fact that maximal stimulation of insulin secretion by ABA requires more time than that observed with glucose (Fig. 2, *B* and *C*) suggests that autocrine ABA may prolong glucose-induced insulin secretion in β cells.

Recently, Guri *et al.* (41) reported that dietary ABA intake improves glucose tolerance in db/db mice fed high fat diets, although the authors did not imply a direct effect of ABA on insulin secretion. Our results, indicating that ABA stimulates insulin secretion and potentiates glucose-induced insulin release, are consistent with this observation.

Because ABA has been shown to be released also by activated human granulocytes (5) and monocytes (18), paracrine generation of ABA may also play a role in potentiation of insulin secretion under conditions of systemic or localized inflammation. From the data presented here, it cannot be inferred whether ABA may act as a protection against or as an inducer of insulin resistance. Under conditions of acute physical stress, hyperglycemia may induce autocrine ABA release from pancreatic β cells, thereby increasing insulin secretion and improving glucose consumption and insulin-mediated anti-inflammatory effects (42). Under conditions of chronic inflammation, however, prolonged stimulation of β cells by ABA released from inflammatory cells may result in an increased, glucose-independent insulin secretion, eventually leading to insulin resistance (19–22). The basal [ABA] in plasma is ~1 nM (18) and nanomolar concentrations of ABA up-regulate insulin secretion (Figs. 1 and 2); these facts suggest a possible role for ABA in the physiology as well as in the dysregulation of human insulin secretion.

REFERENCES

1. Grill, E., and Himmelbach, A. (1998) *Curr. Opin. Plant Biol.* **1,** 412–418
2. Zocchi, E., Carpaneto, A., Cerrano, C., Bavestrello, G., Giovine, M., Bruzzone, S., Guida, L., Franco, L., and Usai, C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14859–14864
3. Zocchi, E., Basile, G., Cerrano, C., Bavestrello, G., Giovine, M., Bruzzone, S., Guida, L., Carpaneto, A., Magrassi, R., and Usai, C. (2003) *J. Cell Sci.* **116,** 629–636
4. Puce, S., Basile, G., Bavestrello, G., Bruzzone, S., Cerrano, C., Giovine, M., Arillo, A., and Zocchi, E. (2004) *J. Biol. Chem.* **279,** 39783–39788
5. Bruzzone, S., Moreschi, I., Usai, C., Guida, L., Damonte, G., Salis, A., Scarfì, S., Millo, E., De Flora, A., and Zocchi, E. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 5759–5764
6. Lee, H. C., Walseth, T. F., Bratt, G. T., Hayes, R. N., and Clapper, D. L. (1989) *J. Biol. Chem.* **264,** 1608–1615
7. Lee, H. C. (ed) (2002) *Cyclic ADP-Ribose and NAADP: Structures, Metabolism and Functions*, pp. 1–21, Kluwer Academic Publishers, Norwell, MA
8. Guse, A. H. (2005) *FEBS J.* **272,** 4590–4597
9. Wu, Y., Kuzma, J., Maréchal, E., Graeff, R., Lee, H. C., Foster, R., and Chua, N. H. (1997) *Science* **278,** 2126–2130
10. McCowen, K. C., Malhotra, A., and Bistrian, B. R. (2001) *Crit. Care Clin.* **17,** 107–124
11. Marik, P. E., and Raghavan, M. (2004) *Intensive Care Med.* **30,** 748–756
12. Gearhart, M. M., and Parbhoo, S. K. (2006) *AACN Clin. Issues* **17,** 50–55
13. Charles, M. A., Fanska, R., Schmid, F. G., Forsham, P. H., and Grodsky, G. M. (1973) *Science* **179,** 569–571
14. Sharp, G. W. G. (1979) *Diabetologia* **16,** 287–296
15. Furman, B., and Pyne, N. (2006) *Curr. Opin. Investig. Drugs* **7,** 898–905
16. Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) *Science* **259,** 370–373
17. Islam, M. S. (2002) *Diabetes* **51,** 1299–1309
18. Zocchi, E., Guida, L., Buzzone, S., Scarfì, S., Magnone, M., Basile, G., Benatti, U., De Flora, A., Moreschi, M., Franco, L., and Salis, A. PCT/IB2006/053669 *Fluridone as an Anti-inflammatory Agent* (October 6, 2006)

19. Dandona, P., Aljada, A., and Bandyopadhyay, A. (2004) *Trends Immunol.* **25,** 4–7
20. Shoelson, S. E., Herrero, L., and Naaz, A. (2007) *Gastroenterology* **132,** 2169–2180
21. de Luca, C., and Olefsky, J. M. (2008) *FEBS Lett.* **582,** 97–105
22. Muoio, D. M., and Newgard, C. B. (2008) *Nat. Rev. Mol. Cell. Biol.* **9,** 193–205
23. Federici, M., Hribal, M., Perego, L., Ranalli, M., Caradonna, Z., Perego, C., Usellini, L., Nano, R., Bonini, P., Bertuzzi, F., Marlier, L. N., Davalli, A. M., Carandente, O., Pontiroli, A. E., Melino, G., Marchetti, P., Lauro, R., Sesti, G., and Folli, F. (2001) *Diabetes* **50,** 1290–1301
24. Melzi, R., Battaglia, M., Draghici, E., Bonifacio, E., and Piemonti, L. (2007) *Transplantation* **83,** 167–173
25. Graeff, R., and Lee, H. C. (2002) *Biochem. J.* **361,** 379–384
26. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–252
27. Schubart, U. K., Shapiro, S., Fleischer, N., and Rosen, O. M. (1977) *J. Biol. Chem.* **252,** 92–101
28. Feng, D. D., Luo, Z., Roh, S. G., Hernandez, M., Tawadros, N., Keating, D. J., and Chen, C. (2006) *Endocrinology* **147,** 674–682
29. Liu, D., Zhen, W., Yang, Z., Carter, J. D., Si, H., and Reynolds, K. A. (2006) *Diabetes* **55,** 1043–1050
30. Miguel, J. C., Abdel-Wahab, Y. H., Green, B. D., Mathias, P. C., and Flatt, P. R. (2003) *Biochem. Pharmacol.* **65,** 283–292
31. Morita, K., Kitayama, S., and Dohi, T. (1997) *J. Biol. Chem.* **272,** 21002–21009
32. Boittin, F. X., Dipp, M., Kinnear, N. P., Galione, A., and Evans, A. M. (2003) *J. Biol. Chem.* **278,** 9602–9608
33. Xie, G. H., Rah, S. Y., Kim, S. J., Nam, T. S., Ha, K. C., Chae, S. W., Im, M. J., and Kim, U. H. (2005) (2005) *Biochem. Biophys. Res. Commun.* **330,** 1290–1298
34. Bruzzone, S., Moreschi, I., Guida, L., Usai, C., Zocchi, E., and De Flora, A. (2006) *Biochem. J.* **393,** 697–704
35. Takasawa, S., Akiyama, T., Nata, K., Kuroki, M., Tohgo, A., Noguchi, N., Kobayashi, S., Kato, I., Katada, T., and Okamoto, H. (1998) *J. Biol. Chem.* **273,** 2497–2500
36. De Flora, A., Guida, L., Franco, L., Zocchi, E., Pestarino, M., Usai, C., Marchetti, C., Fedele, E., Fontana, G., and Raiteri, M. (1996) *Biochem. J.* **320,** 665–671
37. Verderio, C., Bruzzone, S., Zocchi, E., Fedele, E., Schenk, U., De Flora, A., and Matteoli, M. (2001) *J. Neurochem.* **78,** 646–657
38. Bacová, Z., Benický, J., Lukyanetz, E. E., Lukyanetz, I. A., and Strbák, V. (2005) *Cell. Physiol. Biochem.* **16,** 59–68
39. Lee, H. C. (2005) *J. Biol. Chem.* **280,** 33693–33696
40. Masgrau, R., Churchill, G. C., Morgan, A. J., Ashcroft, S. J., and Galione, A. (2003) *Curr. Biol.* **13,** 247–251
41. Guri, A. J., Hontecillas, R., Si, H., Liu, D., and Bassaganya-Riera, J. (2007) *Clin. Nutr.* **26,** 107–116
42. Dandona, P., Chaudhuri, A., Mohanty, P., and Ghanim, H. (2007) *Curr. Opin. Clin. Nutr. Metab. Care.* **10,** 511–517