The Extracellular Calcium-Sensing Receptor on Human β-Cells Negatively Modulates Insulin Secretion

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The presence and functional significance of the extracellular calcium-sensing receptor (CaR) on human pancreatic β-cells were investigated. Reverse transcriptase–polymerase chain reaction with primers for the extracellular domain of the CaR expressed in human parathyroid-secreting cells identified a product of the expected size in human pancreatic mRNA. Immunocytochemistry using an antibody against the extracellular region of CaR showed extensive immunoreactivity in insulin- and glucagon-containing cells but not in somatostatin-containing cells. In perifusion experiments, elevations in extracellular Ca^{2+} produced initial transient increases in insulin secretion, followed by a concentration-dependent and prolonged, but reversible, inhibition of secretion. Microfluorometric measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in isolated human β-cells demonstrated that elevations in extracellular Ca^{2+} (0.5–10 mmol/l) caused rapid elevations in $[Ca^{2+}]_i$. Increases in extracellular Ca^{2+} caused small increases in the cyclic AMP content of whole human islets. These studies demonstrated that human β-cells express an extracellular CaR and that activation of the receptor inhibits basal and nutrient-stimulated insulin secretion. The transduction mechanism that mediates this inhibitory effect is unknown, but our results suggest that it is unlikely to be through the adenylate cyclase-cyclic AMP pathway or through the phopholipase C–IP_3 pathway. This CaR-mediated inhibitory mechanism may be an important autoregulatory mechanism in the control of insulin secretion. *Diabetes* 49:409–417, 2000

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BDA, British Diabetic Association; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium; CaR, calcium-sensing receptor; CMRL, Connaught Medical Research Laboratories; DAG, diacylglycerol; IBMX, isobutylmethylxanthine; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase–polymerase chain reaction; VDCC, voltage-dependent Ca^{2+} channels.

The identification and cloning of an extracellular calcium-sensing receptor (CaR) in the parathyroid gland (1,2) were crucial steps in understanding how cells and organs respond appropriately to fluctuations in extracellular Ca^{2+}. It has since become apparent that the ability to detect changes in extracellular Ca^{2+} is not confined to cells involved in the systemic regulation of plasma Ca^{2+}, such as parathyroid (1), bone (3), and kidney cells (4), and CaR expression has been reported recently in tissues as diverse as fibroblasts (5), antral gastrin cells (6), epithelial cells (7), and oligodendrocytes (3).

The function(s) of CaR in tissues that are not involved in the regulation of plasma Ca^{2+} homeostasis is as yet uncertain, but there is evidence that these cells express CaR to enable them to respond to localized, and often acute, changes in their extracellular Ca^{2+} concentration. For example, we demonstrated recently that human antral gastrin cells use CaR to stimulate gastrin release in response to increases in extracellular Ca^{2+} of dietary origin (6). Furthermore, it has been suggested that CaR expression on neuronal cells is crucial in the regulation of cell function in a microenvironment in which the local extracellular Ca^{2+} can vary rapidly (3,8). CaR has limited homology with the metabotropic glutamate receptor family (9), and it consists of a seven-transmembrane–spanning domain, similar to those in the G-protein–coupled receptor superfamily, that is coupled to a large extracellular domain involved in Ca^{2+}-binding (10). Point mutations of this extracellular domain can markedly affect the affinity of the CaR for Ca^{2+} (11), which may be important when considering potential roles for CaR in the local regulation of cellular function in response to changes in extracellular Ca^{2+}.

Insulin-secreting β-cells in pancreatic islets of Langerhans store insulin in membrane-bound secretory granules, which are rapidly mobilized for exocytosis in response to nutrient or nonnutrient stimuli (12,13). It has been known for many years that β-cell secretory granules also contain very high concentrations of divalent cations, particularly Ca^{2+} and Mg^{2+} (12), although the reasons for this have never been explained fully. The exocytotic release of insulin in response to physiological stimuli is accompanied by the release of the other contents of the secretory granule, including divalent cations. In this study, we have examined whether CaR activation by extracellular Ca^{2+} may act as a local regulator of insulin secretion from pancreatic β-cells.

RESEARCH DESIGN AND METHODS

Preparation of islets of Langerhans. Islets used in this study were supplied by the Dixon's Human Islet Project (King's College Hospital, London) and the Human Islet Facility (University of Leicester, Leicester, U.K.). Briefly, pancreases were removed (with permission) from nondiabetic, heart-beating cadaver organ donors, and islets of Langerhans were isolated under aseptic conditions by use of a method similar to that described previously (14). Islets were maintained (37°C, 95:5% air:CO_2) in Connaught Medical Research Laboratories (CMRL) culture medium supplemented with 15% fetal calf serum and 100 U/ml penicillin with 0.1 mg/ml streptomycin. All tissue culture reagents were obtained from Gibco (Paisley, U.K.). Pancreases used in polymerase chain reaction (PCR) and immunocytochemistry studies were collected in collaboration with the British Columbia Transplant Society, and ethical permission was granted from the University of British Columbia Clinical Screening Committee.

Reverse-transcriptase–PCR. mRNA was isolated from human pancreas and antrum by use of a commercially available kit (Micro mRNA Purification Kit; Pharmacia Biotechnology, Quebec, Quebec, Canada). Random hexamer-primed first-strand cDNA was prepared from 300 ng of mRNA per reaction, and PCR amplification was performed with oligonucleotide primers designed to amplify a 374-bp region of the extracellular domain of the human parathyroid hormone–secreting cell CaR (6). The sequences of forward and reverse primers were CTACATTCC CCAGGTCAGTT (nucleotides 918–937) and GGACAATCACCTTGATGAGG (nucleotides 1272–1291), respectively. To increase reaction stringency, the touchdown method of PCR was used (15). The initial annealing temperature of 66°C was reduced to 56°C for standard PCR cycling. PCR products were resolved by electrophoresis in a 2% agarose gel.

Immunocytochemistry. Human pancreas, fixed in Bouin's solution for 1 h, was washed in 70% ethanol, dehydrated, and embedded in paraffin wax at 60°C. Sections 5-µm thick were cut and dewaxed with xylene (2 × 2-min wash) and cleared in petroleum ether (2 × 5-min wash). Sections were incubated with monoclonal anti-CaR (ADD; NPS Pharmaceuticals, Salt Lake City, UT) at 1:500 in 5% horse serum and phosphate-buffered saline (PBS) with 0.01% Triton X-100 overnight at 4°C. The antibody was removed, and the sections were washed three times in PBS and 0.01% Triton X-100 for 5 min before being incubated in Alexa 594 goat anti–mouse IgG conjugate (Molecular Probes, Eugene, OR) at 1:500. For double staining the CaR, immunostained sections were reincubated overnight at 4°C in one of the following solutions: rabbit anti-somatostatin (Dako, Copenhagen) at 1:1,000 in PBS with 0.01% Triton X-100 and 5% horse serum; rabbit anti-glucagon (Sigma, St. Louis, MO) 1:500; or guinea pig anti-insulin (obtained from Dr. Pederson, University of British Columbia, Vancouver, BC, Canada) 1:1,000. The rabbit antibodies were localized with Alexa 488 goat anti–mouse IgG conjugate (Molecular Probes), and the guinea pig antibodies were localized with fluorescein isothiocyanate–conjugated rabbit anti–guinea pig IgG at 1:500 (Miles Labs, Bethesda, MD). Sections were imaged using an Micro Radiance Confocal 600 microscope (Bio-Rad, Mississauga, ON, Canada). The image stacks were processed using National Institutes of Health Image (share ware) software (Bethesda, MD), and final images were produced using Adobe Photoshop (Adobe Systems, Klamath Falls, OR).

Single-cell microfluorometry. Pancreatic islets were dispersed by mild agitation in trypsin, and cells were plated onto 3-aminopropyltriethoxysilane–coated (Sigma) glass coverslips and were allowed to adhere overnight in CMRL under standard tissue culture conditions. Cells were loaded for 20 min at 37°C with 2.5 µmol/l of the Ca^{2+}-fluorophore Fura-2/AM (Sigma). The coverslips were washed and placed in a steel chamber, the volume of which was ~500 µl. A single 22-mm coverslip formed the base of the chamber, which was mounted into a heating platform on the stage of an Axiovert 135 Research Inverted microscope (Carl Zeiss, Welwyn Garden City, U.K.). All experiments were carried out at 37°C using a Na^+-rich balanced salt solution as the standard extracellular medium (16). A low-pressure rapid superfusion system (flow rate 1–2 ml/min) was used to change the solutions in the bath. Cells were illuminated alternatively at 340 and 380 nm with an Axon Imaging Workbench (Axon Instruments, Foster City, CA). Emitted light was filtered using a 510-nm long-pass barrier filter and was detected by using a Photonics Science ISIS camera (Roberts-Bridge, Sussex, U.K.). Changes in the emission intensity of Fura-2 expressed as a ratio of dual excitation were used as an indicator of changes in $[Ca^{2+}]_i$ by use of established procedures (17). Although calibration procedures are subject to error (18), these ratio values (R) can be used to estimate actual $[Ca^{2+}]_i$ according to the following formula (19):

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

where the dissociation constant for Fura-2, K_d = 225 nmol/l (17), and R_{min}, R_{max}, and β are constants (0.41 ± 0.05 [n = 8], 3.11 ± 0.76 [n = 10], and 4.41 ± 0.66 [n = 8], respectively). These constants have been determined using in situ calibration procedures described by Schlegel et al. (19,20). Data were collected every 3 s for multiple regions of interest (up to 25) in any one field of view. All records have been corrected for background fluorescence (determined from cell-free coverglass).

Insulin secretion. Groups of islets (500–1,000) were loaded into chambers and perifused (0.5 ml/min, 37°C) with a physiological salt solution (23) supplemented with 2 mmol/l glucose, 0.5 mmol/l $CaCl_2$, and 0.5 mg/ml bovine serum albumin (BSA), essentially as described previously for rat islet (21). After a 30-min equilibration period, perifusate samples were collected at 2-min intervals for the duration of the experiments and stored at –20°C until analysis. The concentration of $CaCl_2$ and/or glucose in the perifusion solution was varied during the experiments, as described in RESULTS. Islets were retrieved from the supporting filters at the end of the experiment, and insulin was extracted by the addition of acidified ethanol and sonication. Insulin content of perifusate samples and islet extracts was measured by radioimmunoassay, as described previously (22).

Cyclic AMP measurement. Islets were washed twice by gentle centrifugation (1,000g, 2 min) and resuspended in a physiological salt solution (23) supplemented with 2 mmol/l glucose, 0.5 mmol/l $CaCl_2$, and 0.5 mg/ml BSA. After preincubation (30 min, 37°C), groups of islets (100–300) were dispensed into 1.5-ml microcentrifuge tubes and pelleted by centrifugation (1,000g, 2 min). The supernatant was removed, and islets were resuspended and incubated (20 min, 37°C) in 1 ml of the salt solution supplemented with 2 or 20 mmol/l glucose and various concentrations of $CaCl_2$ in the presence or absence of 100 µmol/l isobutylmethylxanthine (IBMX). Islets were repelleted by centrifugation (1,000g, 2 min), and the supernatant was removed and stored at –20°C to await assay for insulin content. The islet pellet was disrupted by sonication in acetate buffer (50 mmol/l sodium acetate, pH 6.2) and heated to 100°C for 3 min, and the resulting extracts were stored at –20°C until assayed for cyclic AMP content by radioimmunoassay, as described previously (24).

Protein measurement. Groups of islets (10–20) were washed twice with PBS and pelleted by centrifugation (1,000g, 2 min). The pellet was lysed by the addition of 0.5 mol/l NaOH and sonication. Protein content of the samples was measured by use of Bradford's protein assay (25).

Data analysis. Differences between means were assessed by analysis of variance, Student's t test, or Bonferroni's test for multiple comparisons, as appropriate, and considered significant when $P < 0.05$.

RESULTS

Expression of CaR mRNA in human pancreas. Human pancreas expresses an mRNA species that can be amplified by using PCR primers for the parathyroid CaR receptor cDNA, as shown in Fig. 1. The pancreatic product is similar to that amplified from mRNA extracted from human antrum, a tissue known to express the CaR (6). Both products yielded the expected DNA fragment of ~374 bp (Fig. 1), as confirmed by restriction cut digests using *Eco*RV and *Rsa*I (data not shown).

Expression of CaR protein in human islet cells. Immunocytochemical staining by use of a monoclonal antibody to the human parathyroid cell CaR detected ~90% of the cells within human pancreatic islets, but no CaR-immuno-

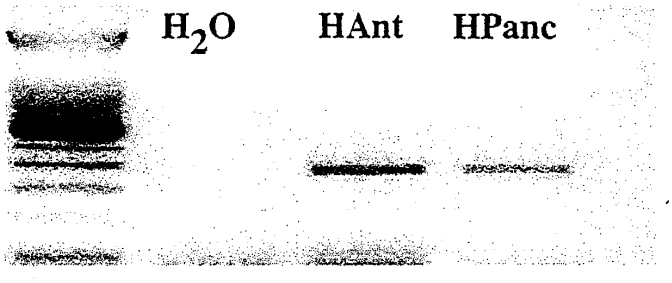

FIG. 1. Detection of CaR mRNA in human pancreas. Products obtained from RT-PCR amplification of mRNA from human pancreas and antrum after separation by agarose gel electrophoresis are shown. The HPanc column contains a PCR product from a human pancreatic mRNA sample. The HAnt column contains a PCR product from human antral (HAnt) preparation, and the H_2O column contains a PCR product from the control in the absence of the template. The products in the HAnt and HPanc columns were of the expected size (374 bp) for the fragment of the parathyroid hormone–secreting cell CaR. The column furthest to the left shows a standard mRNA ladder.

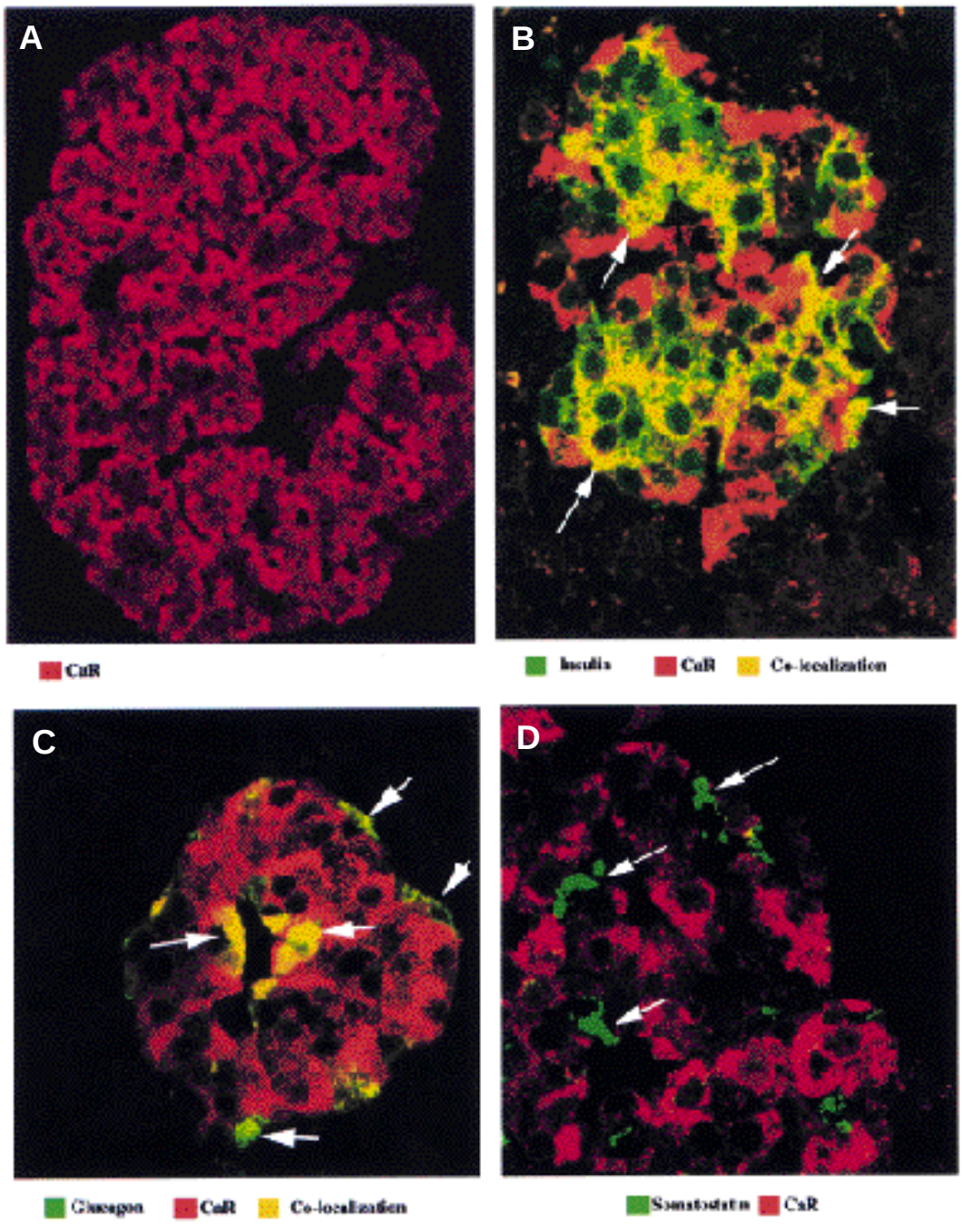

FIG. 2. Immunostaining of a human islet for the CaR and islet hormones. *A*: Immunocytochemical staining of the CaR within a 5-µm section of a human islet. *B*: Insulin-immunoreactive cells, double-stained with the antibody to the CaR. Note the extent of co-localization (yellow shading). *C*: A different islet double-stained for glucagon and the CaR, showing extensive co-localization (lower left panel). *D*: A third islet stained for somatostatin and the CaR (lower right panel). Note that somatostatin-immunoreactive cells are not co-localized to CaR-staining.

reactivity was detected in pancreatic exocrine tissue (Fig. 2*A*). Dual immunostaining by use of antisera for islet hormones demonstrated that the CaR receptor was expressed on human β- and α-cells, because ~90% of the islet cells were also immunoreactive for insulin or glucagon (Fig. 2*B* and *C*). However, CaR immunoreactivity was not detected in islet cells that also stained for somatostatin (Fig. 2*D*), suggesting that the CaR receptor was not expressed on human δ-cells. Absorption of the CaR antibody with the immunogen blocked the immunostaining, but it did not interfere with the binding of the insulin, glucagon, or somatostatin antibodies.

Insulin secretion from human islets. The insulin and total protein content of the human islets used in these studies was 4.8 ± 1 ng/islet and 0.4 ± 0.1 µg/islet, respectively (six donors). The pattern of glucose-induced insulin secretion from perifused human islets is shown in Fig. 3. Increasing the glucose concentration in the perifusate from 2 (0–10 min) to 20 mmol/l (10-min end) caused a rapid increase in insulin secretion in the presence of 0.5 mmol/l extracellular Ca^{2+}. In other experiments, glucose-induced insulin secretion was further enhanced by the adenylate cyclase activator, forskolin (10 µmol/l forskolin, 184 ± 34% 20 mmol/l glucose secretion, $P < 0.01$).

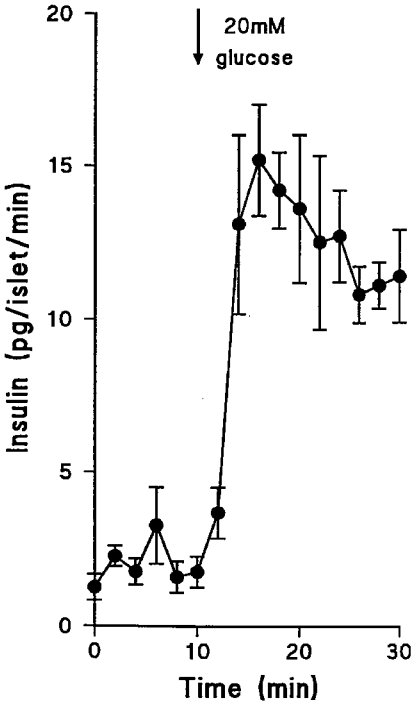

FIG. 3. Glucose-induced insulin secretion from human islets. Increasing the glucose concentration in the perifusate from 2 mmol/l (0–10 min) to 20 mmol/l (10–30 min) caused a rapid increase in insulin secretion from human islets with secretion declining to initial levels despite the continued presence of the stimulatory concentration of glucose. Data are means ± SE of three separate perifusion channels.

Effects of extracellular Ca^{2+} on insulin secretion. At a substimulatory concentration of glucose (2 mmol/l), increasing extracellular Ca^{2+} from 0.5 to 5.0 mmol/l caused an initial and rapid increase in insulin secretion from perifused islets, followed by a marked inhibition of secretion below basal levels (Fig. 4*A*). The inhibition of secretion was maintained when the extracellular Ca^{2+} concentration was increased to 10 mmol/l. Similar concentration-dependent inhibitory effects of extracellular Ca^{2+} were observed at a stimulatory concentration of glucose (20 mmol/l) (Fig. 4*B*). However, under these conditions, increasing extracellular Ca^{2+} to 5 mmol/l caused a smaller transient increase in secretion than that seen in the presence of 2 mmol/l glucose (Fig. 4*A*). The extracellular Ca^{2+}-induced inhibition of insulin secretion was fully reversible at both glucose concentrations; the secretory rate rapidly returned to normal when the extracellular Ca^{2+} concentration was reduced to 0.5 mmol/l (Fig. 4*A* and *B*).

Effects of extracellular Ca^{2+} on $[Ca^{2+}]_i$ in human β-cells. The mean basal cytosolic Ca^{2+} level was estimated to be 75 ± 9 nmol/l (means ± SE of 36 cells) when extracellular Ca^{2+} was 0.5 mmol/l. As shown in Fig. 5*A*, at 0.5 mmol/l extracellular Ca^{2+}, individual human β-cells responded to the sulfonylurea tolbutamide (100 μmol/l) and to direct depolarization by KCl (20 mmol/l) with rapid increases in $[Ca^{2+}]_i$. The mean basal-to-peak amplitude of these responses was 265 ± 26 nmol/l (32 cells from nine experiments in four donors) and 427 ± 41 nmol/l (10 cells from five experiments in two donors), respectively. In tolbutamide-responsive cells, elevating extracellular Ca^{2+} from 0.5 to 5.0 mmol/l at substimulating concentrations of glucose (2 mmol/l) evoked a rapid increase in $[Ca^{2+}]_i$ (26 of 32 cells from nine experiments

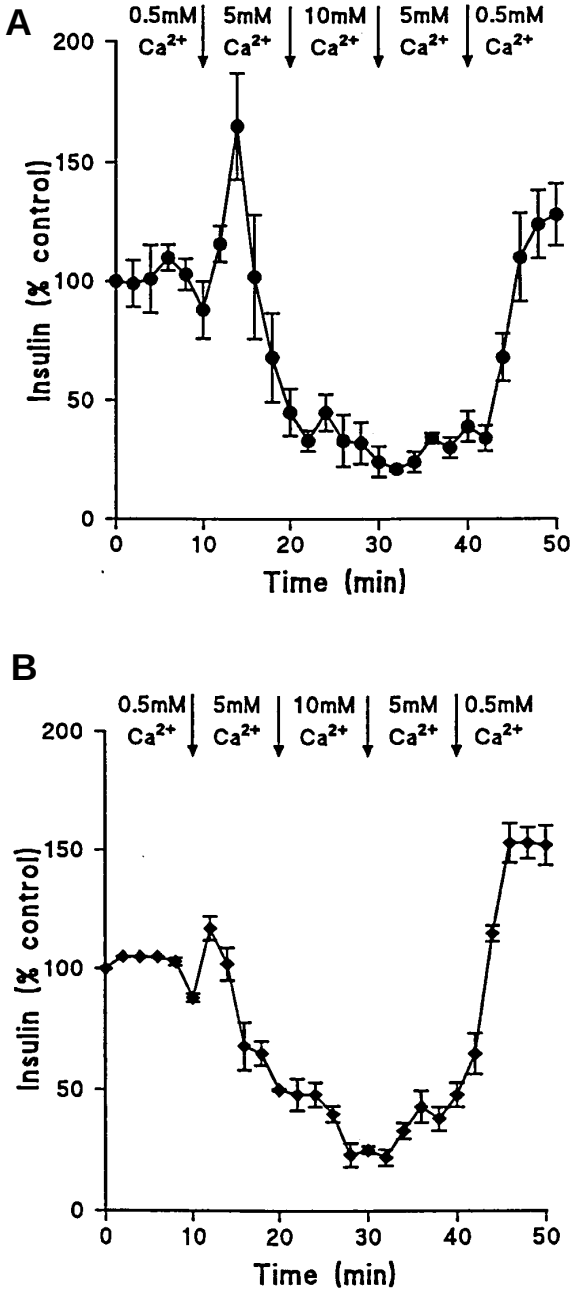

FIG. 4. Effects of changes in extracellular Ca^{2+} on insulin secretion. *A*: Human islets were perifused with solutions containing 2 mmol/l glucose and various concentrations of Ca^{2+}. Increasing the extracellular Ca^{2+} concentration caused a reversible inhibition of insulin secretion. *B*: Similar effects were observed when the perifusate contained 20 mmol/l glucose. Data are means ± SE from three separate perifusions and are typical of results from three separate donors. Secretion is expressed as a percentage of the control insulin release in the presence of 0.5 mmol/l extracellular Ca^{2+}.

in four donors). The mean basal-to-peak amplitude of the Ca^{2+}-induced response was 43% of the mean tolbutamide response (113 ± 14 nmol/l, 26 cells from nine experiments in four donors). The profile of the Ca^{2+}-induced increase in $[Ca^{2+}]_i$ showed both intra- and interdonor variability (Fig. 5*B*). In 39% (10 of 26) of those cells examined, the Ca^{2+}-evoked rise in $[Ca^{2+}]_i$ had returned to basal levels within 3 min of the original application, even in the continued presence of

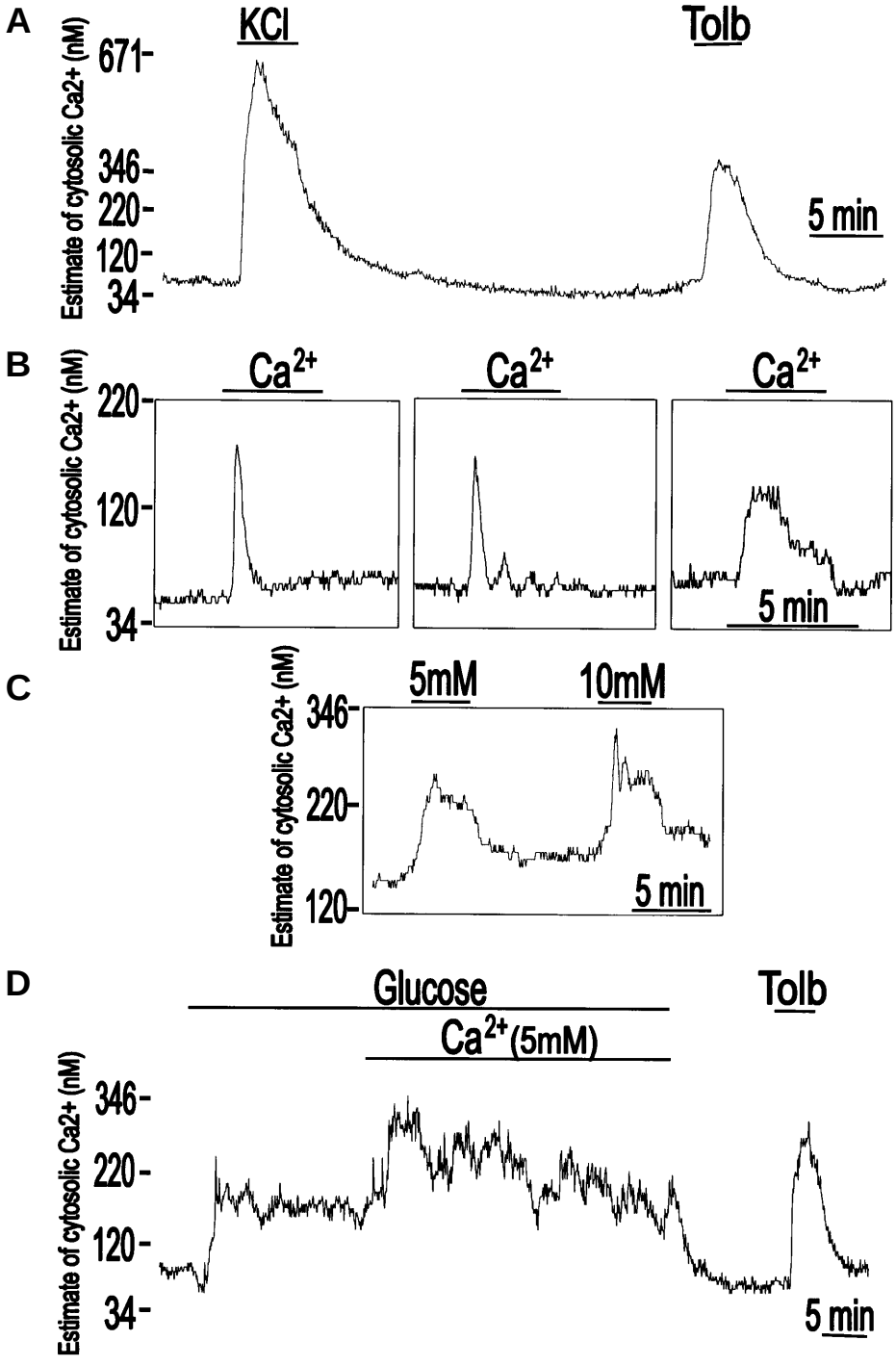

FIG. 5. Effects of changes in extracellular Ca^{2+} on intracellular Ca^{2+}. *A*: A representative trace of tolbutamide (100 µmol/l)- and KCl (20 mmol/l)-evoked changes in $[Ca^{2+}]_i$ in cells dispersed from human islets. *B*: At substimulatory concentrations of glucose (2 mmol/l), increasing extracellular Ca^{2+} from 0.5 to 5.0 mmol/l evoked an increase in $[Ca^{2+}]_i$. Data represent the responses in three cells from separate donors. *C*: In the same cell, increasing extracellular Ca^{2+} from 0.5 to 5.0 or 10 mmol/l evoked a concentration-dependent increase in $[Ca^{2+}]_i$. *D*: At 0.5 mmol/l extracellular Ca^{2+}, increasing the glucose concentration from 2 to 20 mmol/l evoked a sustained increase in $[Ca^{2+}]_i$. The amplitude of this response was accentuated when the concentration extracellular Ca^{2+} was elevated to 5 mmol/l in the continued presence of stimulatory concentrations of glucose. Data are from three separate experiments.

elevated concentrations (5 mmol/l) of extracellular Ca^{2+}. The response to extracellular Ca^{2+} is concentration-dependent (Fig. 5*C*). At the single-cell level, it was noted that no gross morphological changes resulted from elevated concentrations of extracellular Ca^{2+}, and the effects of raised extracellular Ca^{2+} were fully and rapidly reversible. In tolbutamide-unresponsive cells, 17% (2 of 12) exhibited an increase in $[Ca^{2+}]_i$ in response to elevated extracellular Ca^{2+}. At 0.5 mmol/l extracellular Ca^{2+}, increasing the glucose concentration from 2 to 20 mmol/l evoked a sustained increase

TABLE 1
Cyclic AMP content of human islets of Langerhans

Treatment	Cyclic AMP (% basal)	Insulin (% basal)
0.5 mmol/l Ca^{2+}	100	100
5.0 mmol/l Ca^{2+}	159 ± 18*	51 ± 2†
10 mmol/l Ca^{2+}	128 ± 16‡	52 ± 2†
10 µmol/l forskolin	728 ± 115†	77 ± 8‡

Data are means ± SE (n = 4) and are typical of experiments using tissue from two different donors. Groups of human islets of Langerhans were incubated, as described in RESEARCH DESIGN AND METHODS, and cyclic AMP content and insulin secretion into the medium were assayed by radioimmunoassay. Under basal conditions (2 mmol/l glucose, 0.5 mmol/l $CaCl_2$), islets contained 1.03 ± 0.12 fmol/l cyclic AMP per islet and secreted 0.2 ± 0.02 ng insulin · islet^{-1} · 20 min^{-1}. *P < 0.05 vs. control; †P < 0.001 vs. control; ‡P > 0.2 vs. control.

in $[Ca^{2+}]_i$ in tolbutamide-responsive cells. Increasing extracellular Ca^{2+} under these glucose-stimulated conditions resulted in a further rise in cytosolic Ca^{2+} (Fig. 5*D*).

Effects of extracellular Ca^{2+} on cyclic AMP in human islets. The cyclic AMP content of human islets was significantly increased by incubation in the presence of the adenylate cyclase activator, forskolin (Table 1), or the phosphodiesterase inhibitor, IBMX (100 µmol/l IBMX, 172 ± 20% control, P < 0.05). Note, however, that forskolin-induced elevations in cyclic AMP did not initiate insulin secretion from the human islets in the presence of a substimulatory concentration of glucose (2 mmol/l) (Table 1), which is in accordance with cyclic AMP acting to potentiate secretory responses initiated by nutrients (26). Table 1 also shows that, in the presence of 2 mmol/l glucose, increasing extracellular Ca^{2+} produced small elevations in islet cyclic AMP content. Measurements of insulin secretion in the same experiments confirmed that secretion was significantly inhibited by elevations in extracellular Ca^{2+} (Table 1). Similar results were obtained in experiments performed in the presence of a stimulatory concentration of glucose (20 mmol/l glucose and 10 mmol/l Ca^{2+}; cyclic AMP content 173% vs. control; insulin secretion 43% vs. control).

DISCUSSION

Ca^{2+} is a pivotal intracellular signal in the regulation of insulin secretion from pancreatic β-cells. The initiation of insulin secretory responses by nutrient secretagogs is dependent on an influx of extracellular Ca^{2+} across the plasma membrane through L-type voltage-dependent Ca^{2+} channels (VDCC) (27,28). Modulation of nutrient-induced insulin secretion by receptor-operated agonists, such as peptide hormones and neurotransmitters, often involves the activation of phospholipase C and the consequent generation of IP_3 and the release of Ca^{2+} from intracellular stores (29). The results of the present study suggest that Ca^{2+} may also play an important role in the regulation of insulin secretion by acting as an extracellular autocrine messenger that regulates insulin secretion through interaction with an extracellular CaR on the β-cell.

Our results leave no doubt that human primary β-cells express the CaR. Thus, reverse transcriptase (RT)-PCR amplification of the CaR mRNA in cDNA prepared from human pancreatic tissue clearly demonstrated pancreatic transcription of the CaR gene, whereas the CaR protein was localized by immunocytochemistry to the endocrine pancreas, rather than the majority of the exocrine organ. Even within the endocrine organ of the islet of Langerhans, there was cell-specific expression of the CaR protein; expression was associated with insulin-secreting β-cells and glucagon-secreting α-cells, but not with somatostatin-secreting δ-cells. These observations may suggest a specific function for the CaR in β- and α-cells, rather than a ubiquitous function in peptide hormone-secreting cells in general. Our observations in human primary tissue are supported by a recent study in which RT-PCR identified CaR mRNA in human insulinoma tissues (30), although neither the expression of the CaR protein nor its functional significance on insulin secretion was addressed in that study.

Any interpretation of the role of changes in extracellular Ca^{2+} in a putative CaR-mediated regulation of insulin secretion from β-cells is complicated by two factors: *1*) the importance of an influx of extracellular Ca^{2+} into β-cells down a concentration gradient as a mechanism for elevating intracellular $[Ca^{2+}]_i$ (27,28); and *2*) the obligatory role of elevations of $[Ca^{2+}]_i$ in the initiation of β-cell secretory responses to depolarizing nutrient stimuli (27,28). These factors suggest that experimental increases in extracellular Ca^{2+}, by means of increasing the Ca^{2+} concentration gradients across the β-cell plasma membrane, should lead inevitably to increases in $[Ca^{2+}]_i$ and, thus, to increased insulin secretion; this is largely what has been reported in studies using tissues isolated from experimental animals (27,28,31–35). In accordance with these observations, increasing extracellular Ca^{2+} did cause elevations in $[Ca^{2+}]_i$ in human β-cells in our experiments, and this effect was associated with a rapid, though transient, increase in insulin secretion from perifused islets, a finding that is consistent with early reports in rodent islets (34) and perfused pancreases (36). In parathyroid hormone–secreting cells, the mobilization of stored Ca^{2+} by CaR agonists, such as spermine or neomycin, is transitory (37), a finding again consistent with the Ca^{2+}-induced changes in cytosolic Ca^{2+} reported in the present study. The enhancement of insulin secretion was more marked at 2 mmol/l glucose than at 20 mmol/l glucose; this finding is consistent with insulin secretion being caused by changes in $[Ca^{2+}]_i$, which was already elevated in the presence of the stimulatory concentration of glucose, as demonstrated by our measurements of glucose-induced changes in $[Ca^{2+}]_i$.

However, and unexpectedly, the overall effect of elevating extracellular Ca^{2+} was a marked inhibition of insulin secretion from human islets. Thus, after the initial rise in insulin secretion, increased extracellular Ca^{2+} caused a concentration-dependent reduction in the secretory rate, both under basal conditions and in the presence of a stimulatory glucose concentration; this finding is similar to those of early reports of the inhibitory action of elevated extracellular Ca^{2+} on insulin secretion in rabbit pancreas (38). The Ca^{2+}-induced inhibition was reproducible within and between islet preparations from different donors, and it was rapidly reversible upon lowering extracellular Ca^{2+} to more physiological concentrations, suggesting that it was not a generalized toxic response of the β-cells to Ca^{2+}. In accordance with this, individual β-cells showed normal $[Ca^{2+}]_i$ responses to the sulfonylurea, tolbutamide, after prior exposure to 5 mmol/l extracellular Ca^{2+}. A simple explanation for these observations is that elevations in extracellular Ca^{2+} result in activation of the CaR, which is

expressed on human β-cells, leading to an inhibition of insulin secretion. This hypothesis could be tested by using a specific agonist of the CaR, such as the phenylalkylamine derivative NPS R-568 (39), but to date, these compounds are not commercially available. However, polyvalent cations, such as magnesium (Mg^{2+}), cobalt (Co^{2+}), and gadolinium (Gd^{3+}), and polycationic compounds, such as polyamines, have been used in other cell systems to activate the CaR (40–42). In pancreatic islets, spermine inhibits glucose-induced insulin-secretion (43), whereas spermidine and putrescine, which are less potent agonists of the CaR (42), had no effect on glucose-induced secretion. We have previously shown that polymyxin B, another polyamine, has a profound inhibitory effect on insulin secretion (44), whereas the inhibitory action of the divalent cation Co^{2+} can be dissociated from any inhibition of Ca^{2+} influx (45) and, thus, is consistent with activation of the CaR.

It seems inherently unlikely that β-cells express the CaR to detect changes in circulating concentrations of Ca^{2+}. Plasma Ca^{2+} is tightly regulated and will not normally approach the concentrations used in our experiments to activate the β-cell CaR. In addition, there is no obvious physiological rationale for β-cells to modify insulin release in response to the small physiological fluctuations in plasma Ca^{2+}. Therefore, we suggest that β-cells express the CaR as an autocrine-sensing system for the regulation of insulin secretion. Patch-clamp capacitance measurements suggest that stimulated β-cells exhibit a very rapid initial rate of exocytosis of primed secretory granules (13), and insulin-containing granules contain high concentrations of the divalent cations Ca^{2+}, Mg^{2+}, and Zn^{2+} (12). The calculated localized increase in the extracellular Ca^{2+} concentration within 1 μm of the β-cell surface will be ~2.5 mmol/l if the following conditions are assumed: *1*) a mean β-cell diameter of 10 μm (46); *2*) a mean granule diameter of 300 nm (12); *3*) an intragranular Ca^{2+} concentration of 120 mmol/l (12); *4*) an initial granule release rate of 500 granules/10 s (13); and *5*) an initial distribution of the exocytosed Ca^{2+} in an extracellular unstirred water layer that extends no more than 10% of the cell diameter. This calculation is supported by the direct measurements of changes in intraislet extracellular Ca^{2+} using a Ca^{2+}-sensistive microelectrode, which detected overall changes in the millimolar (0.5 mmol/l) range in stimulated islets (47). The CaR is also responsive to divalent cations other than Ca^{2+}, and an increase in the localized β-cell extracellular Ca^{2+} will be accompanied by a calculated increase of ~1.5 mmol/l Mg^{2+} and 0.5 mmol/l Zn^{2+}, based on their reported secretory granule concentrations of 70 and 20 mmol/l, respectively (12). Together, the Ca^{2+}, Mg^{2+}, and Zn^{2+} released from insulin-containing secretory granules could produce localized increases in the extracellular concentration of divalent cations of ~5 mmol/l in addition to the preexisting and fairly static concentrations of 1- to 2-mmol/l cations in extracellular fluid. Our results suggest that extracellular cation concentrations of this order are sufficient to inhibit insulin secretion from human β-cells within that microenvironment. Furthermore, the polarity of β-cells in which secretory granules are located preferentially at one pole of the cell (48) could further localize granular Ca^{2+} release and amplify local changes in extracellular Ca^{2+}.

Nutrient-induced insulin secretion from the perfused pancreas is typically biphasic, with a first phase of secretion that increases rapidly, peaks within a few minutes, and then declines rapidly. This is followed by the second phase, which rises more slowly and is maintained at plateau levels for the duration of the stimulus (49). Measurements in single β-cells suggest that the very rapid initial rate of granule exocytosis (13) is not maintained, and the overall average rate of exocytosis from stimulated β-cells is much less than the initial burst of exocytosis (46). This pattern of exocytosis, and its translation into a biphasic pattern of insulin secretion from the whole organ, could be explained by the existence of an autoinhibitory feedback mechanism, in which initial rapid release of an autoinhibitory substance has a negative, concentration-dependent, and reversible effect on further secretion. Our results support the notion that the autoinhibitory feedback is provided by intragranular Ca^{2+} acting on the β-cell secretory mechanism via the extracellular CaR. Thus, in this model, once the stimulatory effects of glucose and the inhibitory effects of local Ca^{2+} fluctuations, via the CaR, have reached equilibrium, the second phase of insulin secretion would attain a sustainable plateau, which would be less pronounced than that seen under first-phase conditions.

The intracellular mechanisms through which activation of the CaR inhibits insulin secretion are unknown, but our results suggest that we can rule out several of the transduction pathways known to be important in β-cells. The inhibitory effects were clearly not caused by a reduction in $[Ca^{2+}]_i$ as demonstrated by our direct measurements of $[Ca^{2+}]_i$. In other tissues, the CaR has been linked to inhibitory effects on the adenylate cyclase-cyclic AMP signaling system (50,51), but this mechanism cannot account for the Ca^{2+}-induced inhibition of insulin secretion from human islets, because we observed that increased extracellular Ca^{2+} actually caused small increases in cyclic AMP accumulation. In some tissues, the CaR is coupled to phospholipase C, and CaR activation generates IP_3 and diacylglycerol (DAG) by phospholipid hydrolysis (3,52). However, neither of these pathways is likely to cause inhibitory effects in β-cells, and the generation of IP_3 and/or DAG is associated with stimuli that increase insulin secretion (29). CaR activation has also been linked to hyperpolarization of lens epithelial cells (7) and gastric mucosal cells (53). Hyperpolarization of β-cells will inhibit their secretory function (54), but this effect cannot explain our results because the CaR-induced inhibition of insulin secretion was observed both in polarized β-cells under resting conditions and in β-cells that were depolarized by 20 mmol/l glucose. Thus, increased extracellular Ca^{2+} enhanced the glucose-induced rise in $[Ca^{2+}]_i$, in accordance with Ca^{2+} entering the depolarized β-cell down its concentration gradient through VDCC (27,28). However, under these conditions, the major effect of increased extracellular Ca^{2+} was to inhibit insulin secretion.

Several inhibitory agonists, including α_2-adrenergic agonists (24), somatostatin (55), and galanin (56), inhibit insulin secretion from β-cells at a late stage in the exocytotic pathway that occurs after the depolarization of the β-cell and the rise in $[Ca^{2+}]_i$; this stage is often referred to as the "distal inhibitory site" (57). Studies using electrically permeabilized islets and β-cells have shown that these agonists can inhibit insulin secretion even when intracellular Ca^{2+} is maintained at micromolar concentrations (24,55). This inhibition is mediated through one or more *pertussis* toxin-sensitive heterotrimeric G-proteins, but the precise identity of the G-protein(s) and the molecular mechanisms linking receptor acti-

vation to inhibition of secretion are presently unknown. A wide range of G_i/G_0 α-subunits are expressed in islets and β-cell lines (57). Transient overexpression of $G_{\alpha i-1}$, $G_{\alpha i-2}$, $G_{\alpha i-3}$, or $G_{\alpha 0-2}$ inhibits Ca^{2+}-induced insulin secretion (58), suggesting that one or more of these subtypes may be involved in receptor-operated agonists acting at the distal inhibitory site. A similar mechanism linking CaR activation to the distal inhibitory site could explain the observed inhibition of insulin secretion by elevated extracellular Ca^{2+}, in spite of associated increases in $[Ca^{2+}]_i$.

In conclusion, our studies using human primary islets of Langerhans suggest that intragranular Ca^{2+} released from stimulated β-cells may modulate further insulin secretion through activation of the extracellular CaR. This mechanism could explain the patterns of exocytosis reported for β-cells and the biphasic pattern of insulin secretion from the pancreas into the circulation

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