Activation of the extracellular calcium-sensing receptor initiates insulin secretion from human islets of Langerhans: involvement of protein kinases

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Abstract

The extracellular calcium-sensing receptor (CaR) is usually associated with systemic Ca^{2+} homeostasis, but the CaR is also expressed in many other tissues, including pancreatic islets of Langerhans. In the present study, we have used human islets and an insulin-secreting cell line (MIN6) to investigate the effects of CaR activation using the calcimimetic R-568, a CaR agonist that activates the CaR at physiological concentrations of extracellular Ca^{2+} . CaR activation initiated a marked but transient insulin secretory response from both human islets and MIN6 cells at a sub-stimulatory concentration of glucose, and further enhanced glucose-induced

insulin secretion. CaR-induced insulin secretion was reduced by inhibitors of phospholipase C or calcium-calmodulin-dependent kinases, but not by a protein kinase C inhibitor. CaR activation was also associated with an activation of p42/44 mitogen-activated protein kinases (MAPK), and CaR-induced insulin secretion was reduced by an inhibitor of p42/44 MAPK activation. We suggest that the β -cell CaR is activated by divalent cations co-released with insulin, and that this may be an important mechanism of intra-islet communication between β -cells.

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Introduction

Since the original identification and cloning of an extracellular calcium-sensing receptor (CaR) in the parathyroid gland (Brown *et al.* 1993), it has become apparent that the ability to detect changes in extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) is not confined to cells involved in the systemic regulation of plasma Ca^{2+} . Thus, CaR expression has now been detected in a wide range of tissues, including neurons and oligodendrocytes (Chattopadhyay *et al.* 1998), pancreatic acinar cells (Bruce *et al.* 1999), ductal epithelium in breast (Yamaguchi *et al.* 2000), hematopoietic precursor cells (House *et al.* 1997), fibroblasts (McNeil *et al.* 1998), and the α - and β -cells in pancreatic islets of Langerhans (Rasschaert & Malaisse 1999, Squires *et al.* 2000).

The physiological function(s) of the CaRs in tissues, which are not involved in the regulation of plasma Ca^{2+} homeostasis, is not completely understood, but CaR expression may allow these cells to detect localized changes in the extracellular Ca^{2+} concentration in their immediate environment. For example, it has been suggested that CaR expression on neuronal cells regulates cell function in a micro-environment in which the local extracellular Ca^{2+} can

vary rapidly (Hofer *et al.* 2000); that CaR in the exocrine pancreas monitors Ca^{2+} in pancreatic juice to reduce the risk of formation of calcium carbonate stones (Bruce *et al.* 1999); and that antral gastrin cells utilize CaR to stimulate gastrin release in response to an increase in extracellular Ca^{2+} of dietary origin (Ray *et al.* 1997).

We have demonstrated previously that insulin-secreting β -cells in human islets of Langerhans express CaR, and we proposed that CaR activation by Ca^{2+} and other divalent cations that are co-released with insulin may act as a local regulator of insulin secretion (Squires *et al.* 2000). Those studies used supra-physiological levels of $[\text{Ca}^{2+}]_o$ to activate CaR but, given the importance of an influx of $[\text{Ca}^{2+}]_o$ in the initiation of insulin secretion, it is difficult to ascribe the effects of elevated $[\text{Ca}^{2+}]_o$ solely to CaR activation. In the present study, we have used both the human islets and the MIN6 mouse insulin-secreting cell line to study the effects on insulin secretion of CaR activation using the calcimimetic R-568, a phenylalkylamine CaR agonist that activates CaR by allosterically increasing the affinity of the receptor for Ca^{2+} and other divalent cations, such that CaR can be activated without using non-physiological increases in $[\text{Ca}^{2+}]_o$.

Materials and Methods

Materials

MIN6 cells were provided by Dr Y Oka and Professor J I Miyazaki (Osaka, Japan) (Miyazaki *et al.* 1990). Dulbecco's modified Eagle's medium (DMEM), gelatin, PBS, and EDTA (0.02% solution) were purchased from Sigma. Other tissue culture reagents were obtained from Invitrogen. PCR primers were prepared in-house (Molecular Biology Unit, King's College London), and all other molecular biology reagents were from Promega. The rabbit anti-CaR antibody was raised by Genosphere Biotechnologies (Paris, France) against a synthetic peptide comprising residues 18–29 of the mouse CaR (CSAYGPDQRAQK). 4',6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes (Eugene, OR, USA). The calcimimetic R-568 was a gift from Amgen Incorporation (Thousand Oaks, CA, USA). The monoclonal antibody against p42/44 mitogen-activated protein kinases (MAPK, 1:5000 final dilution) and the rabbit polyclonal antibody against phosphorylated MAPK (1:1000 final dilution) were from BD Bioscience (Cowley, UK). The HRP-labeled goat anti-mouse antibody (1:10 000 final dilution) and HRP-labeled goat anti-rabbit anti-serum (1:5000 final dilution) were from Pierce (Rockford, IL, USA). The protein kinase inhibitor staurosporine (SP), the calcium-calmodulin-dependent protein kinase (CAMK) inhibitor KN-93, the protein kinase C (PKC) inhibitor Go6976, the p42/44 MAPK inhibitor 2'-amino-3'-methoxyflavone (PD98059), and the phospholipase C (PLC) inhibitor 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl]-1*H*-pyrrole-2,5-dione (U-73122) were from Calbiochem. All inhibitors were dissolved in dimethylsulphoxide (DMSO) such that the final concentration of DMSO in the incubation buffers was 0.1%. Controls received 0.1% DMSO alone.

Experimental tissues

Human islets were provided with appropriate informed consent by the Human Islet Transplant Unit at King's College London. Briefly, pancreata were retrieved from non-diabetic heart-beating cadaver organ donors and islets were isolated under aseptic conditions as described (Huang *et al.* 2004).

Islets were maintained (37 °C, 95:5% air:CO₂) in Connaught Medical Research Laboratories (CMRL) culture medium supplemented with 15% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin.

The mouse insulin-secreting MIN6 cell line (passage 35–45) was maintained (37 °C, 95:5% air:CO₂) in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin as adherent monolayers on standard tissue culture plastic, or as non-adherent pseudoislets on gelatin-coated (1% w/v) tissue culture plastic, as described (Hauge-Evans *et al.* 1999). To maintain the integrity of extracellular receptors adherent monolayer cells were retrieved by non-tryptic dissociation using a 0.02% EGTA solution.

CaR expression

CaR mRNA expression was analyzed in MIN6 cells, MIN6 pseudoislets, mouse primary islets, and other mouse tissues by reverse transcription (RT)-PCR, essentially as described (Squires *et al.* 2000). The CaR cDNA was amplified by 40 cycles of PCR (2 mM Mg²⁺, annealing temperature 58 °C) using primers designed to amplify a product of 414 bp (forward: 5'-CACTGCGGCTCATGCTTTCAC-3'; reverse: 5'-GCCTGGTGTCTGTTCAAAGTG-3'). PCR products were resolved by electrophoresis on a 1.8% agarose gel, visualized by ethidium bromide staining and excised from the gels for restriction digestion or for sequencing using standard fluorescent chain-terminator methods. RT-PCR amplification of mRNA isolated from single human islet cells was performed as described earlier (Ramracheya *et al.* *in press*) using the primer sequences and PCR conditions detailed in Table 1.

The expression and distribution of CaR immunoreactivity in MIN6 cells were assessed by fluorescence immunocytochemistry, as described earlier (Squires *et al.* 1999, 2000), using a polyclonal rabbit anti-CaR serum (1:1000), and ALEXA 488-conjugated chicken anti-rabbit IgG (1:2000, 1 h). In some experiments, insulin immunoreactivity was also identified using a polyclonal guinea pig anti-insulin serum (1:1000) and ALEXA 594-conjugated goat anti-guinea pig IgG (1:2000). Nuclear staining was achieved by incubation with 1 µM DAPI for 3 min at room temperature.

Table 1 Single cell RT-PCR. PCR primers, annealing temperatures, and predicted product sizes for RT-PCR amplification of (pre)pro-insulin and CaR from single human islet cells

	Sequences	Annealing temperature (°C)	Product size (bp)
Product			
(pre)pro-insulin (outer)	5'-ccctctggggacctgacc-3' 5'-acaatgccagcttctgc-3'	56	232
(pre)pro-insulin (inner)	5'-aacgaggctcttctcacac-3' 5'-ggtacagcattgttcaca-3'	56	158
CaR (outer)	5'-ctacattccccaggtcagtt-3' 5'-ggacaatctccttgatagg-3'	60	374
CaR (inner)	5'-cagactctcagcaacaagaa-3' 5'-atgctggatctcttcctcatc-3'	58	250

Hormone secretion

Insulin secretion from human islets or from MIN6 pseudoislets was measured using a multichannel perfusion apparatus maintained in a 37 °C temperature-controlled room, as described earlier (Hauge-Evans *et al.* 2002, Al-Majed *et al.* 2004). Perifusate fractions were collected every 2 min and insulin and glucagon contents, as appropriate, were determined by RIA (Jones *et al.* 1988).

Measurement of MAPK activation

Suspensions of MIN6 cells (1×10^6 cells/500 μ l) were incubated (37 °C, 5 min) in a physiological salt solution in the presence or absence of 1.3 mM CaCl₂ and 1 μ M R-568. Cells were pelleted by centrifugation (10 000 g, 1 min), the supernatant was discarded, and protein extracts were prepared as described (Gyles *et al.* 2001). Proteins were separated by PAGE, transferred to membranes and immunoprobed for p42/44 MAPK and for phosphorylated (activated) MAPK, as described (Gyles *et al.* 2001).

Results

CaR expression in human β -cells

Detection of CaR expression by RT-PCR analysis of cDNA prepared from human islets demonstrated expression of a product of the expected size, as shown in Fig. 1A. To confirm that CaR mRNA was expressed within β -cells, RNA samples prepared from single human islet cells were analyzed by RT-PCR. The specificity and selectivity of the technique are shown in Fig. 1B, which represents an experiment where isolated cells from human islets were screened to determine whether they were β -cells by amplifying (pre)pro-insulin (PPI) mRNA. No product was observed in control reactions, which lacked the reverse transcriptase (lane 2) or when 0.5 μ l of the last PBS wash was used instead of an isolated cell (lane 1), confirming that the 158 bp product amplified from a β -cell (lane 3) was derived neither from genomic DNA nor from contaminating nucleic acids released from damaged cells into the wash solution. In the experiment shown in Fig. 1C (typical of three), one out of five cells analyzed from dispersed human islets expressed mRNAs for both CaR and (pre)pro-insulin, confirming the expression of the CaR in human β -cells.

CaR activation and insulin secretion from human islets

The CaR agonist R-568 stimulated insulin secretion from isolated human islets in the presence of a sub-stimulatory concentration of glucose (2 mM) as shown in Fig. 2. Exposure to R-568 (0.1 μ M) in the absence of [Ca²⁺]_o (10–20 min) caused a small but significant increase in insulin secretion ($P < 0.01$). However, increasing concentrations of [Ca²⁺]_o (0.2–1.2 mM) in the presence of a fixed

Figure 1 CaR expression in human islet β -cells. Human islet single cell RNA or 0.5 μ l PBS were subjected to RT-PCR, then analyzed by electrophoresis on a 1.8% agarose gel and ethidium bromide staining. (A) CaR mRNA expression in human islets. The PCR was performed over a range of annealing temperatures using the inner pair of primers designed to amplify a product of 250 bp. (B) Single cell RT-PCR specificity. Two rounds of PCR were performed using primers designed to amplify (pre)pro-insulin (PPI) cDNA: lanes 1 and 2 represent negative controls where either no cell (-cell) or no reverse transcriptase (RT) was added to the reaction mixtures respectively. Lane 3 shows the product generated from one isolated cell with the predicted size of 158 bp. (C) Single cell RT-PCR showing that CaR mRNA is expressed in human β -cells. Five isolated islet cells were subjected to RT, and then to two rounds of PCR to amplify both (pre)pro-insulin (PPI) and CaR cDNAs. Lane 3 shows that one cell expressed both PPI and CaR mRNAs.

concentration of R-568 (0.1 μ M) evoked a much larger stimulation of insulin secretion (Fig. 2). The stimulatory effects of R-568 were rapid with insulin secretion increasing within 1 min of exposure, consistent with a receptor-operated event. The stimulation of insulin secretion was transient, with the rate of secretion returning to near basal levels within 15–20 min, as shown in Fig. 2A. The [Ca²⁺]_o concentration-dependent effects of R-568 are clear in Fig. 2B, which shows the integrated insulin secretory responses to R-568 over 20–40 min of the perfusions. The stimulatory effects of R-568 on insulin secretion were caused by CaR activation and could not be attributed to an artefactual response to changing [Ca²⁺]_o because in parallel control experiments in the absence of R-568 increasing [Ca²⁺]_o from 0 to 0.2–1.2 mM had much less effect on insulin secretion (Fig. 2B, solid bars). Exposing the islets to R-568 in the presence of a fixed concentration of [Ca²⁺]_o also induced a transient stimulation of insulin secretion, but this effect was not R-568-concentration dependent over the range 0.1–10 μ M (1.2 mM Ca²⁺ + 0.1 μ M R-568, peak secretion $2910 \pm 113\%$ basal, mean $\pm$ S.E.M., $n = 4$; + 1.0 μ M R-568, 2721 ± 248 ; + 10 μ M R-568, 2923 ± 567 , ANOVA $P > 0.2$). In addition to initiating a secretory response, CaR activation by R-568 in the presence of 1.2 mM [Ca²⁺]_o potentiated glucose-induced insulin secretion from human islets exposed to a supra-maximal stimulatory concentration

Figure 2 CaR activation stimulates insulin secretion from human islets. (A) Human islets of Langerhans were perfused with a buffered salt solution in the absence of $[\text{Ca}^{2+}]_o$ to establish a basal rate of insulin secretion (0–10 min) after which the islets were exposed to R-568 alone (0.1 μM), R-568 (0.1 μM) in the presence of $[\text{Ca}^{2+}]_o$ (0.2–1.2 mM), and $[\text{Ca}^{2+}]_o$ alone, as shown by the bars. To normalize for differences in tissue loading between experiments data are expressed as % basal secretion in the absence of R-568 and of $[\text{Ca}^{2+}]_o$. ■ = 0.2 mM $[\text{Ca}^{2+}]_o$, ◆ = 0.4 mM $[\text{Ca}^{2+}]_o$, ▲ = 0.8 mM $[\text{Ca}^{2+}]_o$, and ● = 1.2 mM $[\text{Ca}^{2+}]_o$. Points show means ± s.e.m., $n = 3$. (B) Insulin secretory responses to changes in $[\text{Ca}^{2+}]_o$ (0.2–1.2 mM) in the absence (solid bars) or presence (open bars) of R-568 (0.1 μM) are expressed as the mean percentage increase in the rate of secretion over basal, and show a $[\text{Ca}^{2+}]_o$ concentration-dependent secretory response to R-568. Bars show means ± s.e.m., $n = 3$, * $P < 0.05$, ** $P < 0.01$ versus secretion in the absence of R-568.

(20 mM) of glucose (20 mM glucose, $430 \pm 52\%$ basal; +R-568, 738 ± 47 , $P < 0.05$, $n = 4$). In the presence of either 2 or 20 mM glucose CaR activation by R-568 (1.2 mM $[\text{Ca}^{2+}]_o$) did not cause the secondary inhibitory phase of the secretory response that is induced by elevating $[\text{Ca}^{2+}]_o$ above physiological concentrations (Squires *et al.* 2000), suggesting that the $[\text{Ca}^{2+}]_o$ -dependent inhibition that we observed in a previous study was not mediated through the CaR. Consistent with our earlier immunohistochemical localization of CaR in both α - and β -cells in human islets (Squires *et al.* 2000), CaR activation by R-568 in the

presence of 0.4 mM $[\text{Ca}^{2+}]_o$ also caused a small but significant stimulation of glucagon secretion from human islets, with 1 μM R-568 increasing glucagon secretion to $232.7 \pm 21.6\%$ of the basal rate of secretion in the absence of R-568 ($P < 0.05$, $n = 3$).

CaR expression in MIN6 cells

MIN6 cells expressed both CaR mRNA and immunoreactive protein. Figure 3A shows the RT-PCR amplification of products from MIN6 cells configured as monolayers or as pseudoislets corresponding to the expected product size as detected in cDNA prepared from mouse islets, kidney, and brain. Restriction digestion (Fig. 3B) and sequencing of the PCR product confirmed its identity. CaR immunoreactivity was consistent with the processing and insertion of a cell surface transmembrane receptor, with punctate staining in the cytoplasm and on the plasma membrane of MIN6 cells (Fig. 4B), and minimal CaR immunoreactivity associated with insulin-immunoreactive secretory vesicles (see overlay image, Fig. 4D).

Effects of CaR activation in MIN6 cells

Supra-physiological increases in $[\text{Ca}^{2+}]_o$ had similar effects on insulin secretion from MIN6 pseudoislets to those that we have previously reported using human islets (Squires *et al.* 2000). Thus, increasing $[\text{Ca}^{2+}]_o$ from 0 to 10 mM induced rapid but

Figure 3 CaR expression in insulin-secreting MIN6 cells. (A) Detection of CaR mRNA in MIN6 cells and primary murine tissue. RT-PCR amplification revealed a single product corresponding to the expected 414 bp fragment of the mouse CaR. Lanes 1, MIN6 cells; 2, mouse islets; 3, mouse kidney; 4, mouse brain; 5, H₂O blank; and 6, molecular weight markers. (B) The RT-PCR product amplified from MIN6 cell mRNA was digested with a single cut using the restriction enzymes *Agel* and *Bsa*MI, producing restriction fragments of the predicted size, as shown by the arrows. Lanes 1, molecular weight markers; 2, CaR PCR product of 414 bp; 3, restriction digest using *Agel* generated products of 141 and 273 bp; 4, restriction digest using *Bsa*MI generated a product of 336 bp; and 5, control digest without restriction enzymes showing unaltered 414 bp CaR product.

Figure 4 CaR immunoreactivity in MIN6 cells. (A) DAPI-localized nuclear staining is shown. (B) The CaR is localized using green ALEXA 488 and demonstrates a punctuate cytosolic and membrane distribution. Insulin immunoreactivity is localized using red ALEXA 594 in (C). After merging (B) and (C), co-stained regions are shown as yellow in (D), demonstrating that minimum CaR immunoreactivity is associated with the insulin-secreting granules.

transient increases in the basal rate of insulin secretion (peak $287 \pm 30\%$ basal, $n=4$, $P<0.01$) followed by a prolonged and reversible inhibition of secretion (nadir, $39 \pm 11\%$ basal, $n=4$, $P<0.01$). However, the activation of CaR by the presence of R-568 (1 μM) at more physiological concentrations of $[\text{Ca}^{2+}]_o$ initiated insulin secretion from the MIN6 pseudoislets in the presence of a sub-stimulatory concentration of glucose (2 mM), as shown in Fig. 5, without the secondary inhibitory phase.

Figure 5 CaR activation stimulates insulin secretion from MIN6 cells. MIN6 pseudoislets were perfused with a buffered salt solution in the absence of $[\text{Ca}^{2+}]_o$ to establish a basal rate of insulin secretion (0–10 min) after which they were exposed to R-568 alone (1 μM) or R-568 (1 μM) in the presence of $[\text{Ca}^{2+}]_o$ (0.2–2.25 mM), as shown by the bars. Data are expressed as percentage of basal secretion in the absence of R-568 and of $[\text{Ca}^{2+}]_o$. ■ = 0.2 mM $[\text{Ca}^{2+}]_o$, ◆ = 0.75 mM $[\text{Ca}^{2+}]_o$, and ▲ = 2.25 mM $[\text{Ca}^{2+}]_o$. Points show means $\pm$ S.E.M., $n=4$.

Increases in $[\text{Ca}^{2+}]_o$ in the absence of R-568 caused small but significant increases in the basal rate of insulin secretion ($+0.2$ mM Ca^{2+} , $192 \pm 19\%$ basal, mean $\pm$ S.E.M., $n=4$, $P<0.05$; $+0.75$ mM Ca^{2+} , $219 \pm 43\%$, $P<0.05$; $+2.25$ mM Ca^{2+} , $269 \pm 38\%$, $P<0.05$), but these responses were much less than those induced by R-568 in the presence of equivalent concentrations of $[\text{Ca}^{2+}]_o$, where the maximum response was over 2000% basal (Fig. 5). Thus, as observed in experiments using human islets (Fig. 2A), exposure of MIN6 pseudoislets to R-568 in the absence of $[\text{Ca}^{2+}]_o$ (10–20 min) caused a small increase in insulin secretion, while increasing concentrations of $[\text{Ca}^{2+}]_o$ (0.2–2.5 mM) in the presence of a fixed concentration of R-568 (1 μM) evoked larger, $[\text{Ca}^{2+}]_o$ concentration-dependent and transient increases in insulin secretion (Fig. 5).

Intracellular mechanisms of CaR activation in β -cells

The intracellular signal transduction pathways linking CaR activation to increased insulin secretion were investigated using pharmacological inhibitors of transduction elements known to be important in β -cells. Figure 6A shows that the R-568 evoked secretory response of MIN6 pseudoislets was inhibited by the PLC inhibitor, U73122 (10 μM), and by the non-selective protein kinase inhibitor, staurosporine (1 μM), although neither treatment totally abolished the effects of R-568. CaR-dependent insulin secretion was also reduced by inhibitors of CAMK (KN-93, 10 μM) or p42/44 MAPK (PD98059, 50 μM), as shown in Fig. 6B. In contrast, an inhibitor of the Ca^{2+} /phospholipid-dependent PKC (Go6976, 1 μM) had no effect on CaR-induced insulin secretion (Fig. 6B).

The p42/44 MAPK enzymes are activated by phosphorylation by an upstream kinase, and the activated form can be detected by immunoblotting using antibodies selective for the phosphorylated form of the enzyme. Figure 6C shows the rapid (5 min) increase in phosphorylated p42/44 MAPK in MIN6 cells exposed to R-568 (1 μM) in the presence of 1.2 mM $[\text{Ca}^{2+}]_o$, while the total p42/44 immunoreactive protein remained unchanged as expected. In parallel experiments, changes in $[\text{Ca}^{2+}]_o$ alone had no detectable effect on the phosphorylation of p42/44 MAPK.

Discussion

Pancreatic β -cells from rodent (Wang *et al.* 1995, Malaisse *et al.* 1999, Rasschaert & Malaisse 1999) and human pancreatic islets (Squires *et al.* 2000), express a receptor that is usually associated with monitoring changes in extracellular Ca^{2+} . There is no physiological rationale to expect insulin-secreting β -cells to respond to fluctuations in plasma calcium, and the function of the β -cell CaR is uncertain. However, the present results using a CaR-activating calcimimetic agent demonstrate that the acute effect of CaR activation in β -cells is a rapid but relatively transient stimulation of insulin secretion, suggesting that CaR expression by β -cells is linked

Figure 6 Intracellular signaling pathways for CaR in β -cells. (A) Insulin secretion in response to R-568 (1 μM) plus $[\text{Ca}^{2+}]_o$ (1.3 mM) is expressed as 100% to demonstrate the degree of inhibition in the presence of either staurosporine (SP; 1 μM) or U73122 (10 μM). Bars show means $\pm$ S.E.M., $n=4$, $^*P<0.01$ versus control. (B) In similar experiments, the presence of either PD98059 (PD; 50 μM) or KN-93 (10 μM) significantly inhibited R-568-induced insulin secretion but Go6976 (1 μM) had no effect. Bars show means $\pm$ S.E.M., $n=4$, $^*P<0.05$ versus control. (C) p42/44 MAPK immunoreactivities (upper panel) and phospho-p42/44 immunoreactivities (lower panel) were detected in extracts of MIN6 cells incubated (5 min, 37 $^{\circ}\text{C}$) as follows: lane 1, in the absence of R-568 and $[\text{Ca}^{2+}]_o$; lane 2, in the presence of R-568 alone (1 μM); and lane 3, in the presence of both R-568 (1 μM) and $[\text{Ca}^{2+}]_o$ (1.3 mM). Arrows show molecular weights calculated from the gel migration positions of proteins of known molecular weights.

to the regulation of the secretory process. The stimulatory effects of CaR activation are in agreement with our previous report of a transient stimulation of insulin release from human islets by elevations in $[\text{Ca}^{2+}]_o$ (Squires *et al.* 2000) and of a calcimimetic enhancing insulin secretion from rodent islets (Straub *et al.* 2000). The stimulatory effects of R-568 on

insulin secretion in the present study cannot be attributed to the Ca^{2+} reintroduction redux that may occur when reintroducing $[\text{Ca}^{2+}]_o$ to cells that have been in a $[\text{Ca}^{2+}]_o$ -free environment (Nemeth 2004), because increasing $[\text{Ca}^{2+}]_o$ in the absence of R-568 did not have any marked stimulatory effect on insulin secretion. The calcimimetic R-568 does not directly activate the CaR but acts to sensitize the CaR to $[\text{Ca}^{2+}]_o$ (Nemeth & Fox 1999), and this is consistent with its effects on insulin secretion in our experiments. Thus, R-568 had little effect on insulin secretion from human islets in the absence of $[\text{Ca}^{2+}]_o$, and the magnitude of the secretory response in the presence of R-568 and $[\text{Ca}^{2+}]_o$ was dependent upon the concentration of $[\text{Ca}^{2+}]_o$, rather than that of R-568, consistent with Ca^{2+} being the agonist at the CaR.

CaR expression in human islets is not confined to β -cells, and the present demonstration found that CaR activation also stimulated glucagon secretion from human islets which supports our previous immunocytochemical localization of CaR on α -cells (Squires *et al.* 2000). These observations complicate interpretation of the insulin secretion data, since it is difficult to discriminate between direct effects of CaR activation on β -cells and the paracrine-stimulatory effects of glucagon on insulin secretion (Ishihara *et al.* 2003). To focus on the direct β -cell effects of CaR activation, we used the mouse MIN6 insulin-secreting cell line configured as islet-like clusters (pseudoislets) to enhance their secretory performance (Hauge-Evans *et al.* 1999, 2002). The MIN6 cells contained the appropriate mRNA for mouse CaR, and expressed immunoreactive CaR, suggesting that they are an appropriate experimental model for primary β -cells. In accordance with this, the insulin secretory responses of MIN6 pseudoislets to CaR activation were similar to those of primary human islets – increasing $[\text{Ca}^{2+}]_o$ to non-physiological concentrations caused an initial rapid increase in insulin secretion, followed by a prolonged inhibition of secretion, as reported for human (Squires *et al.* 2000) and rodent (Malaisse *et al.* 1999) islets. However, in both MIN6 cells and primary human islets CaR activation using R-568 produced the rapid, transient stimulation of insulin secretion, but not the secondary inhibitory phase, perhaps suggesting that the inhibition was a response to non-physiological $[\text{Ca}^{2+}]_o$ rather than a specific effect mediated through the CaR.

It is unusual for receptor-operated stimuli to initiate an insulin secretory response in the absence of a stimulatory concentration of glucose, but CaR activation initiated insulin secretion from human and rodent β -cells without a concomitant nutrient stimulation, consistent with an important function for CaR activation in the regulation of insulin secretion. Our observations that R-568 could further enhance the maximal secretory response of human islets to glucose are consistent with a previous report in rat islets (Straub *et al.* 2000), and imply that CaR activation enhances the exocytotic release of insulin through separate transduction pathways to those used by glucose and other nutrients. There are numerous

reports linking the CaR to a variety of intracellular transduction cascades in different tissues (McNeil *et al.* 1998, Arthur *et al.* 2000, Buchan *et al.* 2001, Godwin & Soltoff 2002), suggesting that the intracellular effector systems through which CaR activation modulates cell function may be tissue-dependent. Our experiments implicate CaR-linked PLC activation generating inositol trisphosphate (IP₃) to liberate Ca²⁺ stored in the endoplasmic reticulum as one transduction mechanism in β -cells. Thus, CaR-dependent insulin secretion was inhibited by a PLC inhibitor although the inhibition was incomplete, which may suggest the additional involvement of other signal transduction systems. The non-selective protein kinase inhibitor, staurosporine, also inhibited CaR-induced insulin secretion, while the use of a more selective kinase inhibitor implicated CAMK II, which is thought to be involved in β -cell secretory responses to a variety of Ca²⁺-mobilizing stimuli (Jones & Persaud 1998, Easom 1999, Rochlitz *et al.* 2000). These observations are consistent with elevation in [Ca²⁺]_i through IP₃-induced increases in [Ca²⁺]_i, or an influx of [Ca²⁺]_o, activating CAMK and thus initiating an exocytotic response. The diacylglycerol (DAG) generated by PLC activation is also thought to play an important role in the stimulation of insulin secretion by receptor-operated stimuli, such as acetylcholine or cholecystokinin, by activating one or more of the DAG-sensitive isoforms of PKC that are expressed in β -cells (Jones & Persaud 1998). However, the PKC inhibitor Go6976 had no effect on the secretory responses to CaR activation, suggesting that the DAG-sensitive PKC isoforms do not play an important role in this system.

In other tissues, CaR activation is associated with the activation of the MAPK transduction cascade (McNeil *et al.* 1998), and our results suggest a similar involvement in β -cells. The p42/44 MAPK enzymes are activated by an upstream dual specificity kinase, and CaR activation in MIN6 cells caused a rapid increase in the phosphorylation of p42/44 MAPK, consistent with MAPK activation being involved in the insulin secretory response. Complete activation of p42/44 MAPK required the presence of both [Ca²⁺]_o and R-568, and the observation that R-568 alone was much less effective is in accordance with the effects on insulin secretion, and suggests a link between p42/44 MAPK activation and the secretory response. This involvement was supported by our observation that an inhibitor of p42/44 MAPK abolished CaR-mediated insulin secretion. The importance of p42/44 MAPK in CaR-induced insulin secretion was somewhat unexpected because previous studies have demonstrated that activation of p42/44 MAPK is not required for nutrient-induced or receptor-induced insulin secretion (Burns *et al.* 1997, Bocker & Verspohl 2001), and that pharmacological activation of p42/44 MAPK is insufficient to initiate an insulin secretory response (Burns *et al.* 1997). Taken together, these observations suggest that the β -cell CaR initiates insulin secretion by a process that is dependent on both the activation of p42/44 MAPK and the activation of other intracellular pathways, including CAMK II activation. The involvement

of p42/44 MAPK in initiating an insulin secretory response to a receptor-operated, G-protein-coupled stimulus comprises a novel β -cell signaling pathway.

The physiological significance of CaR in pancreatic β -cells is unclear, but we suggest it is involved in intra-islet communication. There is considerable evidence that cell-cell communication within the islet of Langerhans is required for the recruitment of β -cells into a full insulin secretory response (Bosco & Meda 1997), although the nature of this communication remains uncertain. We have previously suggested that the β -cell CaR is activated by the divalent cations (Ca²⁺, Mg²⁺, and Zn²⁺) that are present in high concentrations in insulin-secretory granules (Hutton 1989). On exocytosis, these cations are co-released with insulin and may transiently reach sufficient extracellular concentrations to activate the CaR (Perez-Armendariz & Atwater 1986, Squires *et al.* 2000). Our present results demonstrate that the consequence of this activation is a transient but marked stimulation of insulin secretion. The concept of the CaR being used for intercellular communication based on transient changes in [Ca²⁺]_o has also been suggested as a mechanism through which nerve cells co-ordinate their activity (Hofer *et al.* 2000), so the widespread distribution of CaR expression in the central nervous system (Yano *et al.* 2004) and in endocrine organs (Squires 2000) may reflect a common function in cell-cell communication.

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