

Original Paper

Calcium-sensing Receptor Activation Increases Cell-cell Adhesion and β -cell Function

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Key Words

Calcium-sensing Receptor • E-cadherin • Calcimimetic • Cell-coupling • β -cell • Insulin Secretion

Abstract

Background/Aims: The extracellular calcium-sensing receptor (CaR) is expressed in pancreatic β -cells where it is thought to facilitate cell-to-cell communication and augment insulin secretion. However, it is unknown how CaR activation improves β -cell function.

Methods: Immunocytochemistry and western blotting confirmed the expression of CaR in MIN6 β -cell line. The calcimimetic R568 (1 μ M) was used to increase the affinity of the CaR and specifically activate the receptor at a physiologically appropriate extracellular calcium concentration. Incorporation of 5-bromo-2'-deoxyuridine (BrdU) was used to measure cell proliferation, whilst changes in non-nutrient-evoked cytosolic calcium were assessed using fura-2-microfluorimetry. AFM-single-cell-force spectroscopy related CaR-evoked changes in epithelial (E)-cadherin expression to improved functional tethering between coupled cells.

Results: Activation of the CaR over 48hr doubled the expression of E-cadherin (206 \pm 41%) and increased L-type voltage-dependent calcium channel expression by 70% compared to control. These changes produced a 30% increase in cell-cell tethering and elevated the basal-to-peak amplitude of ATP (50 μ M) and tolbutamide (100 μ M)-evoked changes in cytosolic calcium. Activation of the receptor also increased PD98059 (1-100 μ M) and SU1498 (1-100 μ M)-dependent β -cell proliferation. **Conclusion:** Our data suggest that activation of the CaR increases E-cadherin mediated functional tethering between β -cells and increases expression of L-type VDCC and secretagogue-evoked changes in $[Ca^{2+}]_i$. These findings could explain how local changes in calcium, co-released with insulin, activate the CaR on neighbouring cells to help ensure efficient and appropriate secretory function.

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Introduction

The extracellular calcium sensing receptor (CaR) plays a central role in maintaining systemic Ca^{2+} -homeostasis by regulating the secretion of parathyroid hormone and urinary calcium concentration [1]. However, since the identification and characterisation of the receptor [2], it has become increasingly apparent that this cationic ion binding receptor is found on many tissues not associated with the control of plasma $[\text{Ca}^{2+}]$ including; oesophageal [3] and colonic epithelia [4], the cardiovascular system (reviewed in [5]), hypothalamic neurons [6], pancreatic ducts [7] and pancreatic α - and β -cells [8-10]. The functional significance of the CaR in tissue not involved in the control of systemic Ca^{2+} is not fully understood [11, 12]. In exocrine pancreas it has been suggested that the CaR monitors extracellular Ca^{2+} in pancreatic juice to limit the risk of Ca^{2+} carbonate stone formation [13], whilst the receptor may detect changes in levels of dietary Ca^{2+} in gastrin secreting cells of the human antrum [14-16]. However, a more global explanation for the role of the CaR in these disparate tissues could be in its ability to detect local fluctuations in Ca^{2+} , mediating cell-cell coupling, communication and function.

The possibility that local changes in extracellular Ca^{2+} resulting from the efflux of mobilised Ca^{2+} in one cell are sufficient to activate the CaR on an adjacent cell was elegantly demonstrated in a model system of the kidney epithelium [17]. In this novel study, Ca^{2+} -extrusion from fibroblasts was shown to activate the CaR on co-cultured HEK cells to elicit a response [18]. In the pancreas, cell-to-cell communication improves the functional responsiveness of cells and augments insulin secretion [19, 20]. Specific activation of the CaR using calcimimetics [21] enhances insulin secretion from human islets [10]. Secretory granules contain high concentrations of Ca^{2+} which is released upon exocytosis [22]. Recently Ca^{2+} -sensitive microelectrodes have been used to demonstrate that the extracellular spaces surrounding β -cells constitute a restricted domain where Ca^{2+} , co-released during exocytosis reaches millimolar concentrations [23]. These direct measurements support the concept of local 'hot-spots' of extracellular Ca^{2+} which are able to activate the CaR on neighbouring β -cells.

The use of calcimimetics to specifically activate the CaR by increasing receptor affinity for its ligand avoids the many non-specific effects observed when using supra-physiological changes in calcium concentration. Activation of the CaR in β -cells is associated with rapid increases in cytosolic calcium and marked, but transient, increases in insulin secretion [24, 25]. Our earlier work suggests that the CaR can mediate cell-to-cell communication within islets; however, how this communication is facilitated has yet to be resolved. In the present study we confirm CaR expression in our model of homotypic β -cell- β -cell interaction and provide compelling evidence that activation of the receptor increases expression of the epithelial (E) adhesion protein E-cadherin and increases functional tethering between β -cells. These data could explain why the secretory efficiency of the intact islet is far greater than the sum of its composite β -cells when studied in isolation.

Materials and Methods

Materials

MIN6 cells were obtained from Dr. Y.Oka and J.-I. Miyazaki (Univ. of Tokyo, Tokyo, Japan). DMEM, glutamine, penicillin-streptomycin, gelatin (from bovine skin), PBS, foetal bovine serum and trypsin-EDTA were from Sigma-Aldrich (Poole, Dorset, UK). Tissue culture media and plasticware were from Invitrogen Life Technologies (Paisley, UK). Immobilon P membranes (Millipore, Watford, UK), ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK). Anti-fade Citifluor (glycerol/PBS solution: Agar Scientific, Essex, UK). The MEK inhibitor PD98059 and the inhibitor of VEGF receptor 2, SU1498, that inhibits ERKs phosphorylated by growth factors, were from Calbiochem (Nottingham, UK). The calcimimetic R568 was from Amgen Inc (Thousand Oaks, CA, USA). BrdU, Alexa secondaries and Alexa Fluor 594 conjugated anti-

BrdU were from Invitrogen (Molecular Probes, Eugene, Oregon, USA). Antibodies were obtained from Santa Cruz (CA, USA) and Sigma-Aldrich (Poole, Dorset, UK).

Maintenance of MIN6 cells

MIN6 cells (passage 35-40) were maintained at 37°C (95% air/5% CO₂) in DMEM supplemented with 15% foetal calf serum (FCS), 2mM glutamine and 100U/ml penicillin/0.1mg/ml streptomycin (all Sigma Chemical Co. Poole, Dorset). Cells were split when 80% confluent, about every 3-4 days using Trypsin-EDTA. Prior to treatment, cells were placed in fresh DMEM containing glucose (5mmol/l) and low calcium (0.5mM) overnight.

Immunoblotting

Whole-cell proteins were prepared and separated by SDS-PAGE and electroblotted onto Immobilon P membranes as described previously [26]. Membranes were probed with polyclonal antibodies against human β -catenin (Santa Cruz Biotechnology), raised against amino acids 680-781 mapping at the C-terminus of β -catenin, mouse E-cadherin (Sigma UK product code U3254-anti-uvomorulin), mouse CaR raised against a synthetic peptide corresponding to residues 18-29 of the mouse CaR (CSAYGPDQRAQKK; Genosphere Biotechnologies, Paris-France) and human L-Type voltage gated calcium channel (Santa Cruz Biotechnology) raised against amino acids 1661-1900 mapping within an internal region of L-type Ca²⁺ CP α 1D at dilutions of 1:5000, 1:500, 1:1000, and 1:500 respectively in PBS, 0.05% Tween-20. After three 10-min washes (PBS, 0.1% Tween 20), membranes were incubated with the following secondary antibodies (horseradish peroxidase-conjugated), anti-rat (E-Cadherin diluted 1:20,000 in PBS, 0.05% Tween-20), and anti-rabbit secondary (L-Type Voltage gated calcium channel, β -catenin and CaR all diluted at 1:40,000 in PBS, 0.05% Tween-20) for 60 min at 25°C followed by three 10-min washes (PBS, 0.1% Tween 20). Proteins were visualized using enhanced chemiluminescence and exposure to film. Blots were stripped and re-probed with anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:20,000; R&D Systems) to control for protein loading in subsequent densitometry analysis.

Immunocytochemistry

Cells at 80% confluence were fixed with 4% paraformaldehyde (PFA). Following blocking, the nuclear stain 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1mM) was added for 3mins. Cells were then incubated with antibodies against anti- E-cadherin (Sigma), CaR (Genosphere Biotechnologies, Paris-France), β -catenin (Santa Cruz), and L-Type voltage gated calcium channel (Santa Cruz) diluted at 1:100 in PBS-Triton overnight at 4°C. Candidate proteins were visualized using Alexa488 or 594-conjugated secondary antibodies (1:1,000) in PBS-Triton for 1hr at 25°C. Fluorescence was visualized using an Axiovert 200 fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK).

Calcium Microfluorimetry

MIN6 cells were transferred to APES-coated coverslips and allowed to adhere overnight under standard tissue culture conditions. Cells were then either maintained in control media or cultured in with R568 (1 μ M) for 48hrs. To determine agonist-evoked changes in cytosolic calcium, cells were loaded for 30 minutes in 5mM glucose at 37°C with 5 μ M Fura-2/AM (Sigma, UK). Washed coverslips formed the base of a stainless steel bath placed into a heating platform on the microscope stage (Axiovert 200 Research Inverted microscope, Carl Zeiss Ltd., Welwyn Garden City, UK). All experiments were carried out at 37°C using a Na⁺-rich balanced salt solution as the standard extracellular medium (137mM NaCl, 5.4mM KCl, 1.3mM CaCl₂, 0.8mM MgSO₄, 0.3mM Na₂HPO₄, 0.4mM KH₂PO₄, 4.2mM NaHCO₃, 10mM HEPES and 5mM glucose, pH 7.4). A low-pressure rapid-superfusion system (flow rate 1-2ml/min) was used to change the solutions in the bath to allow for the acute addition of the non-nutrient secretagogues ATP (50 μ M), tolbutamide (100 μ M) and KCl (20mM). Cells were illuminated alternatively at 340nm and 380nm using a Metaflour imaging workbench (Universal Imaging Corp Ltd., Marlow, Bucks, UK). Emitted light was filtered using a 510nm long-pass barrier filter and detected using a Cool Snap HQ CCD camera (Roper Scientific). Data was collected at 3-second intervals for multiple regions of interest in any one field of view. All records have been corrected for background fluorescence (determined from cell-free coverslip).

Cell Proliferation

DNA synthesis as a marker of cell proliferation was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) in individual cells using micro-fluorimetric localisation of BrdU-immunoreactivity as described previously [27]. Briefly, MIN6 cells were seeded onto 3-aminopropyl-triethoxysilane (APES) treated cover glass at a density of 30,000 cells/well and left to adhere overnight under standard tissue culture conditions (95% air/5% CO₂, 37°C) in DMEM supplemented with 15% FCS. Cells were then washed in sterile PBS and serum starved overnight in fresh DMEM containing glucose (5mM) and low calcium (0.5mM). As required, PD98059 [27] or SU1498 [28] were applied as a 1hr pre-treatment, before adding the incubation media containing, calcium (0-10mM), calcimimetic (+/-R568 (1 μ M)) and +/-PD98059 or SU1498 (1-100 μ M). All solutions contained BrdU (10 μ M) for the final 2hr incubation. Cells were fixed with 4% paraformaldehyde (PFA) and their DNA denatured using 1M HCl (30min at RT) before incubating in Alexa-594-conjugated anti-BrdU (Molecular Probes, Invitrogen) at 1:200 and storing overnight at 4°C. After repeated washing (PBS /triton (0.01%)) BrdU incorporation was determined using an Axiovert 200 inverted fluorescent microscope (Carl Zeiss, Welwyn Garden City, UK). Six different wells in three separate experiments were used for each treatment and the number of BrdU-positive cells compared to non-BrdU-immunoreactive cells, determined for several (4-6) arbitrary regions/well in 3-4 separate experiments.

Single cell force spectroscopy

Atomic force microscopy-single-cell force spectroscopy (AFM-SCFS) was carried out using the CellHesion@200 module (JPK Instruments, Berlin, Germany) installed on an Eclipse TE 300 inverted microscope (Nikon, USA). The BioCell™ temperature controller (JPK, Berlin, Germany) was incorporated into the stage of the microscope so that all force spectroscopy measurements were performed at a constant temperature of 37°C. The entire system was placed on top of an anti-vibration table (TMC 63-530, USA). As described previously [29], tipless Arrow TL-1 cantilevers (NanoWorld, Switzerland), with force constant 0.03N/m, were coated with poly-L-lysine (25 μ g/ml in PBS, 30min, RT) and then fibronectin (20 μ g/ml in PBS, 2hr, 37°C), in order to capture a single suspended cell. Prior to cell attachment, a single Force-Displacement (F-D) curve was conducted to calibrate the instrument, using the thermal fluctuation method for fluids (JPK Software Release 3.4, Berlin, Germany). Subsequently, SCFS was conducted by bringing the cantilever-bounded cell into contact with an adherent substrate cell until a determined force (0.8nN) is reached. The two cells remained in contact for a set period of time (5sec), while bonding formed. The cantilever was then retracted at a constant speed (5 μ m/sec) and force (nN) versus displacement (μ m) was continuously measured until the cells were completely separated. For each cell, three successive measurements were performed with 30sec interval pause. F-D measurements from multiple cells (approx 40) in separate experiments (n=4) were completed and the maximum unbinding force (nN) and the detachment energy (fjoules) calculated.

Data Analysis

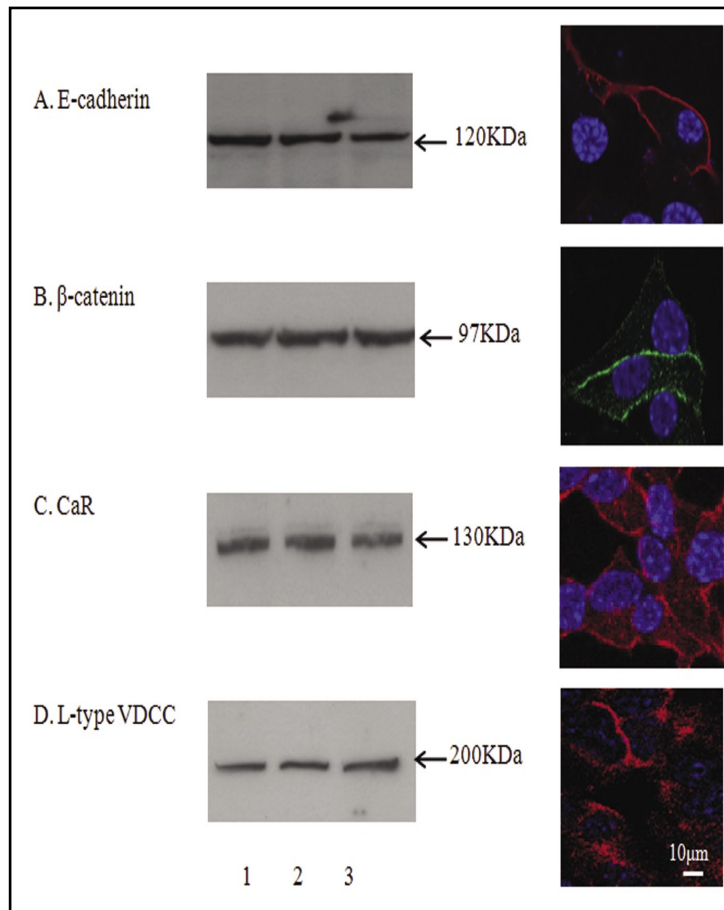
Autoradiographs were quantified by densitometry using TotalLab 2003 (NonLinear Dynamics, Durham, NC USA). In all experiments where data was quantified, the control condition was normalized to 100% and data from all other experimental conditions compared to this. Statistical analysis of data was performed using either a paired t-test or a one-way ANOVA test with a Tukey's multiple comparison post-test. Data are expressed as mean + SEM, and 'n' denotes the number of experiments. $P < 0.05$ was taken to signify statistical significance. All statistical analyses were performed using the Prism GraphPad software version 4.0 (San Diego, CA, USA).

Results

MIN6 cells express the CaR and adherens junction proteins

Western blot analysis confirmed expression of (A) E-cadherin, (B) β -catenin, (C) CaR and (D) L-type VDCC in 3 separate preparations of MIN6 cells, giving rise to bands at 120kDa, 97kDa, 130kDa and 200kDa respectively (Fig. 1). The distribution of each protein was examined by immunocytochemistry. E-cadherin, β -catenin and the CaR exhibited membrane

Fig. 1. MIN6 cells express the CaR and adherens junction proteins. Western blot analyses of MIN6 cell lysates (5 μ g protein/lane) using antibodies against mouse (A) E-cadherin, (B) β -catenin, (C) CaR, and (D) L-type VDCC detected appropriate bands of approximately 120kDa, 97kDa, 130kDa and 200kDa respectively. Controls included antibody pre-absorbed with a 10-fold excess of immunizing peptide (data not shown). Immunocytochemistry confirmed that each of the proteins studied exhibited membrane and cytosolic localization.



and cytosolic localisation, whilst the L-type VDCC appeared more diffusely spread throughout all cell compartments.

Activation of the CaR increases the expression of E-cadherin and L-type VDCCs

MIN6 cells were incubated for 48hrs in low glucose (5mmol/l) with increasing concentrations of extracellular calcium (nominal-no added calcium, 0.5, 5 and 10mM) or 0.5mM calcium \pm the active (R) or inactive (S) stereo-isomers of 568 (1 μ M). Over 48hrs, supra-physiological increases in $[Ca^{2+}]_e$, or addition of the less active S568 isomer of the calcimimetic, failed to alter the expression of any the candidate proteins. Conversely, specific targeting of the CaR by R568 increased whole-cell expression of E-cadherin by $206 \pm 41\%$ of control ($n=4$; $P<0.05$, Fig. 2A) and up-regulated expression of the L-type VDCC to $174 \pm 10\%$ ($n=4$; $P<0.05$) of control (Fig. 2D), but failed to alter the expression of β -catenin (Fig. 2B) or the CaR itself (Fig. 2C). The changes in E-cadherin and L-type VDCC were not observed at earlier time points (e.g. 12 and 24hrs; data not shown).

Activation of the CaR increases adherence between coupled β -cells

In order to relate the change in expression/localisation of E-cadherin to functional tethering, atomic force microscopy-single-cell-force spectroscopy (AFM-SCFS) was used to measure cell-cell adhesion and the separation forces required to uncouple cells. Prior to attachment, cells were cultured for 48hrs under identical conditions +/-R568 (1 μ M). A single MIN6 cell (cell-1) was bound to a cantilever and subsequently brought into contact with an adherent cell (cell-2) within a cluster, using a fixed force (Fig. 3A phase inserts). After 5sec, the cantilever was then retracted (5 μ m/sec) and force versus displacement measured until the cells were completely separated. Retraction force-displacement curves

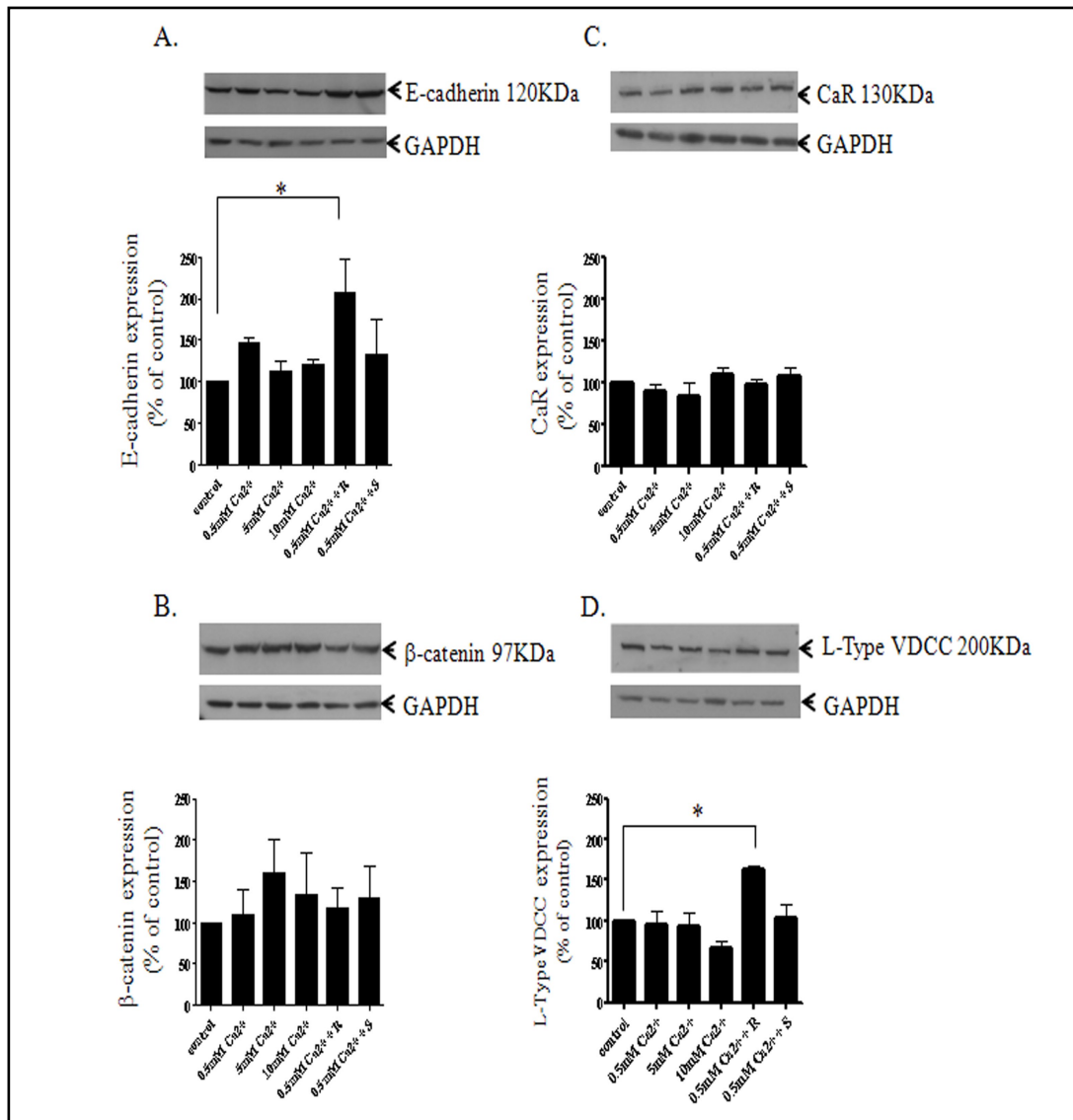
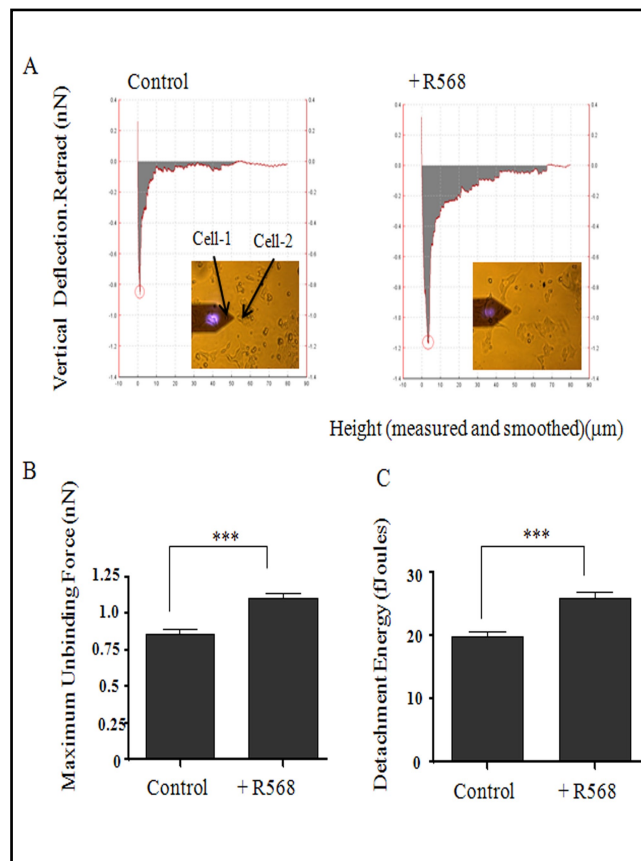


Fig. 2. Activation of the CaR increases expression of E-cadherin and L-type VDCC. MIN6 were grown in low glucose (5mmol/l) +/- calcium (5 or 10mM) or calcium (0.5mM) +/- R568 or S568 (1 μ M) for 48hrs and whole cell expression of (A) E-cadherin, (B) β -catenin, (C) CaR and (D) L-type VDCC was determined by immunoblotting. Upper panels show representative blots for each protein and re-probed for GAPDH as a loading control. Lower panels show mean (\pm SEM) densitometry data, normalised against the non-stimulated low glucose, nominal calcium control (100%), from 3 or more separate experiments. Each bar in the histogram corresponds to the associated lane in the representative blot. Key significances are shown, * $P < 0.05$.

provide important information regarding the adhesion between two cells, such as the energy required to separate them (the grey area in panel A), and maximum force of detachment (red circle). The former is normally referred to as “detachment energy” (panel C) and the latter the “maximum unbinding force” (panel B). The retraction measurements of control (38 cells in 4 separate experiments) and R568-treated MIN6 cells (43 cells in 4 separate experiments) are shown in the figure 3B & C. The results indicate that the calcimimetic R568 (1 μ M) increases the maximum unbinding force by $29 \pm 3.1\%$ ($n=4$; $P < 0.001$), whilst the detachment energy, or the total energy (fjoules) required to uncouple cells following R568 treatment, was increased $31 \pm 3.9\%$ ($n=4$; $P < 0.001$).

Fig. 3. Activation of the CaR reduces functional tethering. AFM-force spectroscopy was used to measure the detachment energy (fJoules) and maximum unbinding force (nN) required to uncouple two MIN6 cells. In panel A, a single MIN6 cell can be seen bound to the cantilever (cell-1, phase insert). This was brought into contact with an adherent cell-2 using a fixed force (1nN) for 10sec, whilst bonding formed. The cantilever was then retracted (5 μ m/sec) and force versus displacement measured until the cells separated (approximate pulling length 10-30 μ m). The energy required to separate the cells (grey area in panel A), and maximum force of detachment (red circle) was measured. The former is known as the "detachment energy" (panel C) and the later is "maximum unbinding force" (panel B). The calcimimetic R568 (1 μ M) increased the maximum unbinding force by 29%, and the detachment energy increased by 31% compared to control. Data is expressed as mean \pm SEM. of multiple cells from 4 separate experiments, where key significances are shown, *** $P < 0.001$.



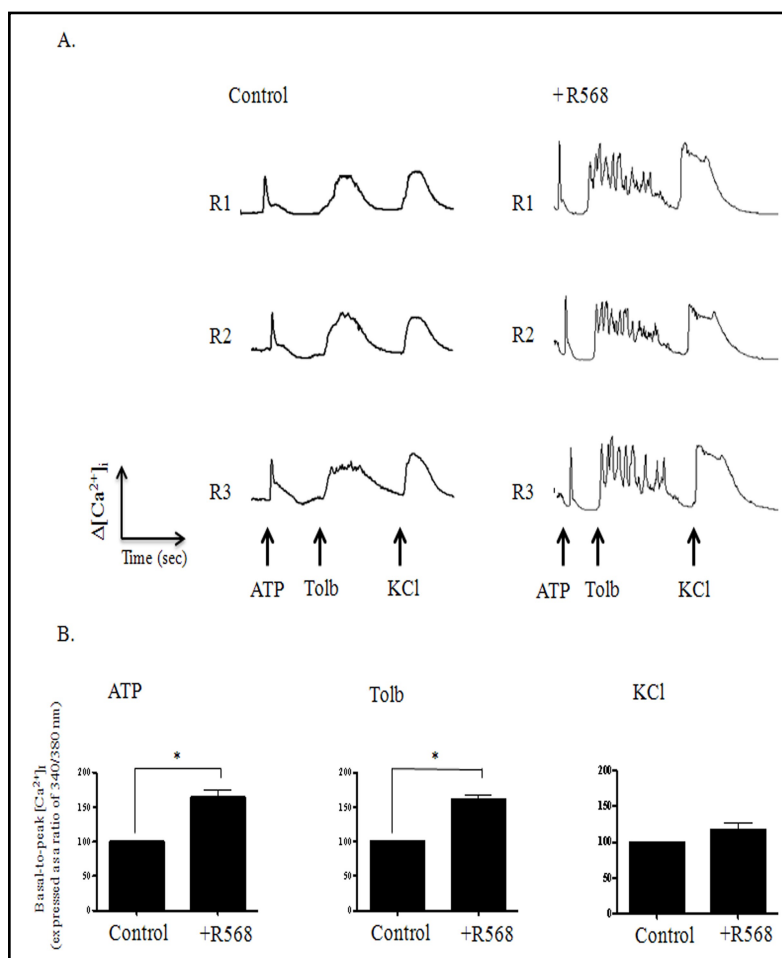
Activation of the CaR alters the amplitude of non-nutrient-evoked $[Ca^{2+}]_i$ oscillations in β -cells

G-protein coupled receptors, such as the P_2 -purinoreceptors, are important modulators of β -cell function, whilst sulphonylureas (such as tolbutamide) stimulate insulin secretion by closing the ATP-sensitive K^+ -channels (K^+_{ATP}) leading to membrane depolarization and Ca^{2+} -entry. To relate the CaR-evoked change in protein expression of E-cadherin and L-type VDCC, the effects of chronic activation of the CaR on these non-nutrient evoked changes in $[Ca^{2+}]_i$ were examined. In figure 4, the effects of ATP (50 μ M), Tolbutamide (100 μ M) and KCl (20mM) on changes in $[Ca^{2+}]_i$ from MIN6 cells cultured in R568 (48hrs, 1 μ M) in low glucose (5mmol/l) is determined. Figure 4A is a representative trace from 3 regions (cells) in a single experiment (R1-3) showing that ATP, tolbutamide and KCl evoked increases in intracellular calcium in both control and R568-treated cells (in a total of five separate experiments). The basal-to-peak change in $[Ca^{2+}]_i$ induced by ATP and tolbutamide were respectively increased to 164 \pm 19% and 161 \pm 12% of control (n=3; $P < 0.05$). The calcimimetic failed to significantly alter the basal-to-peak KCl response.

Activation of the CaR increases MAPK-dependent proliferation

MIN6 cells were incubated in 0.5mM $[Ca^{2+}]_e \pm$ R568 (48hrs, 1 μ M) and BrdU-incorporation was used to determine cell proliferation (Fig. 5A). Low extracellular calcium (0.5mM) increased proliferation compared to nominal calcium conditions (154% \pm 12%). This increase in proliferation was enhanced in the presence of R568 (218 \pm 37% compared to nominal calcium control, n=3 $P < 0.05$, Fig. 5B). It has been previously reported that p42/44 MAPK are active in non-stimulated MIN6 cells. In a separate series of experiments, the R568 (1 μ M) significantly increased proliferation by 171 \pm 18% as compared to 0.5mM Ca^{2+} -alone (n=3 $P < 0.01$). This CaR-evoked increase in cell turnover was dramatically reduced to 29 \pm 5.1%

Fig. 4. Activation of the CaR increases ATP and Tolbutamide-evoked changes in $[Ca^{2+}]_i$. In panel A, ATP (50 μ M), tolbutamide (100 μ M) and KCl (20mM)-evoked changes in $[Ca^{2+}]_i$ in MIN6 cells +/- R568 (1 μ M). The calcimimetic altered the profile of the response to the non-nutrient secretagogues and increased the basal-to-peak amplitude to ATP and the sulphonylurea (panel B). There was no significant change in the amplitude to KCl in response to R568. Key significances are shown, * $P < 0.05$.

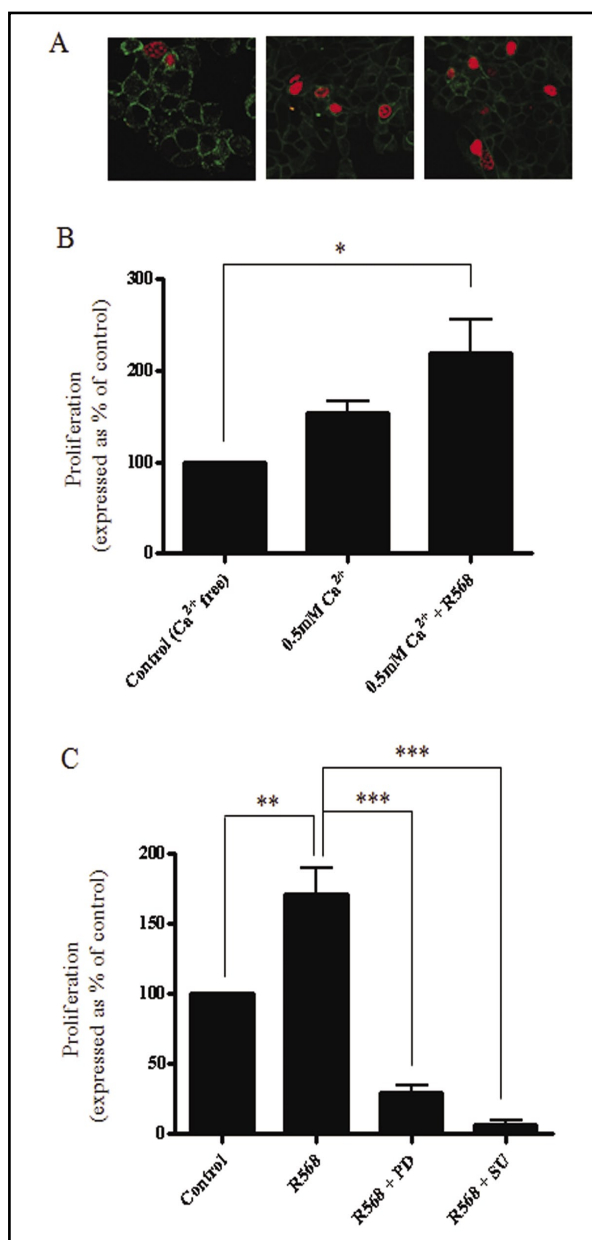


and $7 \pm 2\%$ of the low calcium control by both PD98059 (100 μ M) and SU1498 (100 μ M) respectively ($n=3$ $P < 0.001$, see Fig. 5C). In a further series of experiments ($n=4$), inhibition was found to be concentration-dependent with 1 μ M, 10 μ M and 100 μ M PD98059 reducing proliferation to $86 \pm 10\%$, $52 \pm 1\%$ ($P < 0.01$), and $11 \pm 11\%$ ($P < 0.001$) of 0.5mM calcium alone, whilst 1 μ M, 10 μ M and 100 μ M SU1498 inhibited basal β -cell turnover by $52 \pm 12\%$ ($P < 0.01$), $43 \pm 6\%$ ($P < 0.01$), and $3 \pm 3\%$ ($P < 0.001$) respectively, data not shown.

Discussion

Cadherins are important in forming the multi-protein adherens junction (AJ) that links cell-cell contact to the actin cytoskeleton and various signalling molecules [30]. The extracellular domain mediates ligation with E-cadherin on adjacent cells [31], whilst the cytoplasmic domain binds to β -catenin linking cadherin to the actin cytoskeleton via α -catenin. Interaction of cadherin with F-actin, via the catenins, not only serves to increase adhesive strength of the junction but also acts as a signalling 'node' for proteins that influence adhesiveness &/or initiate intracellular signalling. Co-localised with ECAD and β CAT at the sites of cell-cell contact, connexins (Cx) oligomerise into hexameric hemichannels (connexons) connecting the cytoplasm of adjoining cells and forming gap junctions (GJ). These GJs allow transfer of solutes, metabolic precursors and electrical currents [32], and are essential for synchronising activity across the bulk mass of the islet to ensure effective and appropriate secretion [33]. Inhibiting cadherin-based cell adhesion inhibits GJ-assembly [34], whilst expression of recombinant cadherins into cells lacking strong coupling, increases

Fig. 5. Activation of the CaR increases MAPK-dependent cell proliferation. Cell proliferation was determined by the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into MIN6 DNA and visualized by ALEXA 594-tagged anti-BrdU (red cells, representative panel A). Data represent cell counts from 4-6 arbitrary regions (10-50 cells/region) from 3-4 repeat slides in 3 separate experiments. All data were recorded in the absence of FCS and expressed as a % of a nominal Ca^{2+} control (panel B). Small increases in proliferation induced by low $[\text{Ca}^{2+}]_e$ (0.5 mM) were significantly increased by R568 (1 μM). Inhibition of the p42/44 MAPK pathway using the MEK inhibitor PD98059 (100 μM) or SU1498 (100 μM) reduced proliferation by 71% and 93% respectively (C). Key significances are shown where ** $P < 0.01$, *** $P < 0.001$ for 3 separate experiments.



Cx-phosphorylation at the AJ [35] and increases cell-to-cell communication [36]. Since intercellular adhesion precedes GJ-formation and inhibiting cadherin-based cell adhesion inhibits GJ-assembly, we hypothesize that a gain in cell-cell adhesion will have beneficial effects on β -cell function.

There is considerable evidence suggesting that the extracellular calcium-sensing receptor (CaR) can mediate cell-to-cell communication within the pancreatic islet [8, 10, 24, 25]; however, how this communication is facilitated has yet to be resolved. In the present study we confirm CaR expression in our model of homotypic β -cell- β -cell interaction and report that chronic activation of the receptor increases expression of the epithelial (E) adhesion protein E-cadherin. Furthermore, using single-cell force-spectroscopy we present novel data confirming that the CaR-evoked increase in E-cadherin produces a 30% increase in functional tethering between coupled β -cells. The relationship between the CaR and E-cadherin is consistent with data from other cell types. In colonic cells, regulation of E-cadherin and β -catenin was found dependent on CaR expression and function [37], whilst

inactivation of the CaR inhibited E-cadherin-mediated cell-cell adhesion in human epidermal keratinocytes [38], further supporting our hypothesis that the link between CaR function and AJ proteins can sustain/improve function.

In addition to promoting cell-cell adhesion, chronic activation of the CaR also increased the expression of the L-type voltage-dependent calcium channel (VDCC). Essential for insulin secretion, these channels permit Ca^{2+} -entry ahead of nutrient-evoked exocytosis in the β -cell. Spatial interactions between voltage dependent calcium channels and the CaR have recently been suggested to be important in the first phase of insulin secretion from β -cells [39], and the increased CaR-evoked expression seen in our study could explain the increase in the basal-to-peak amplitude of secretagogue-evoked changes in $[\text{Ca}^{2+}]_i$ in response to both the purinergic agonist ATP, or sulphonylurea activation of the K^+_{ATP} channel by tolbutamide.

There are a number of studies from diverse cell types that suggest a role for the CaR in regulating cell proliferation, including work in fibroblasts [40], astrocytoma [41], and osteoblasts [42]. The low mitotic index of primary β -cells leads to considerable technical difficulties in measuring β -cell proliferation in islets. Previous studies demonstrate that although MIN6 cells are transformed, their proliferative capacity is regulated by extracellular signals, validating these clonal β -cells as a representative experimental model of β -cell proliferation [43-46]. Elevated $[\text{Ca}^{2+}]_e$ can activate p38 and p42/44 MAP kinases [27, 47] and increase cell proliferation [48]. In addition to the effect of chronic activation of the CaR on cell-cell-adhesion we have also shown that specific activation of the CaR by the calcimimetic R568, enhances MEK-dependent proliferation of β -cells at physiologically appropriate concentrations of extracellular calcium.

Conclusion

In the current study we have provided compelling evidence that activation of the calcium-sensing receptor increases expression of the epithelial (E) adhesion protein E-cadherin and increases functional tethering between β -cells. The increase in cell-cell interaction is mirrored by an increase in the expression of the L-type VDCC and augmented secretagogue-evoked changes in $[\text{Ca}^{2+}]_i$. Together, these findings could help explain how local changes in co-released calcium activate the CaR on neighbouring cells to help propagate and synchronise activity within the intact islet to help ensure efficient and appropriate insulin secretion.

Acknowledgements

This work was supported by the generous grant support of Diabetes UK (BDA: 09/0003913). The authors are grateful to Amgen Inc. for the supply of R568 and S568. We would like to thank JPK Instruments for their professional support in the use of single-cell force spectroscopy and we are grateful to Dr MN Hodgkin and Dr GJ Rogers who contributed to some of our early ideas on CaR-activity in the β -cell. The authors have nothing to disclose.

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