Activation of the extracellular calcium-sensing receptor increases functional tethering between pancreatic β-cells

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1. Introduction

The extracellular calcium-sensing receptor (CaR) enables cells to detect and respond to changes in extracellular calcium. Our previous studies have shown that the CaR is expressed by islet β-cells and that pharmacological activation of the islet CaR, using the calcimimetic R568, results in enhanced secretion of insulin.

Epithelial (E)-cadherin (ECAD) is a trans-membrane protein whose extracellular domain forms calcium-dependent homodimers with cadherins expressed on neighbouring cells facilitating cell-to-cell adhesion. This not only serves to increase adhesive strength of the junction but also acts as a signalling 'node' for various proteins that can influence adhesiveness and/or initiate intracellular signalling events. It is well established that a down-regulation of E-cadherin reduces insulin secretion.

We hypothesise that the extracellular calcium-sensing receptor modifies cell-to-cell adhesion within the pancreatic islet and predict that activating the receptor increases ECAD expression and function.

2. Methods

Tissue culture:
MIN6 cells (passage 34-44) were maintained at 37°C (95% O_2/5% CO_2) 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 passaged when 80% confluent every 3-4 days using Trypsin-EDTA. Prior to treatment, Cells were serum starved overnight before applying 0.5mM Ca^{2+} ± R or S568 (1mM) for 48hr in 5.5mM glucose.

Western analysis:
Filters were analyzed with specific polyclonal antibodies against E-cadherin and the CaR in PBS-T (0.05%) overnight at 4°C. Specific proteins were detected using an ECL detection reagent chemiluminescence system.

Immunocytochemistry:
This was performed using standard techniques and protein staining visualised by Alexa fluorescent secondary antibodies (Molecular Probes).

Single cell force spectroscopy*:* Single-cell force spectroscopy (CellHesion® module, JKP Instrument Germany) was used to measure cell-cell adhesion and the separation forces required in uncoupling these cells. A single MIN6 cell was bound to a cantilever using fibronectin (20mg/ml) and poly-l-lysine (25µg/ml) and subsequently brought into contact with an adherent cell (in a cluster of coupled cells) using a known force (1nN). The two cells remained in contact for 10sec whilst bonding formed. The cantilever was then retracted at a constant speed (5µm/sec) and force (nN) versus displacement (deflection of the cantilver) measured using a laser, until the cells were completely separated (pulling length 20-30µm). Each cell-cell recording was repeated in triplicate with a 30sec pause interval between successive measurements. Retraction recordings from multiple cells (approx 50) in separate experiments (n=5) were made and the maximum unbinding force (nN) and the detachment energy (fJoules) calculated.

3. Results: *Expression and localization of CaR and E-cadherin in MIN6 cells:*

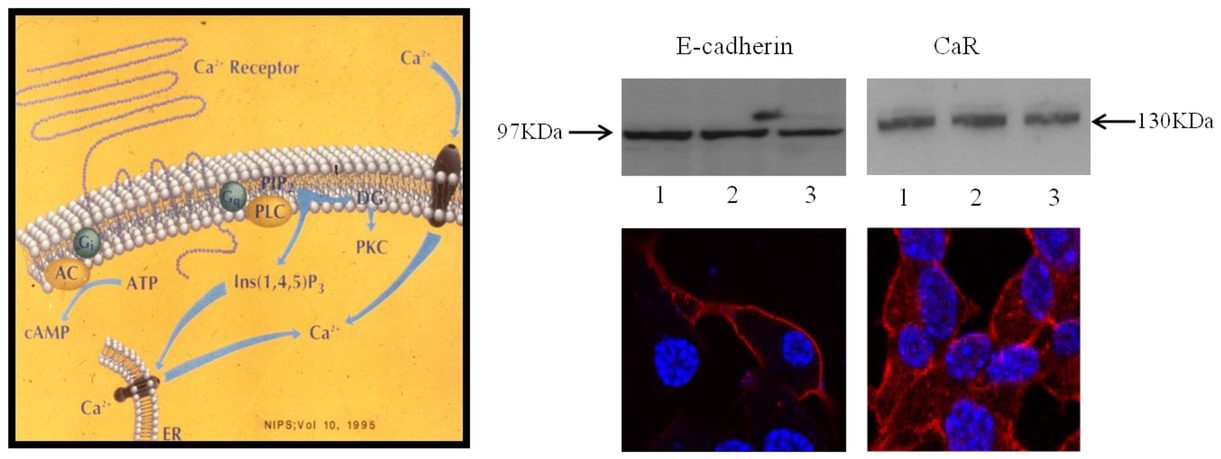


Figure 1: Immunocytochemistry of MIN6 cells and Whole-cell western blot analyses of cell lysates (5µg protein/lane from 3 separate preparations) were visualised using antibodies against E-cadherin and the CaR. Protein bands of approximately 97kDa (ECAD), and 130kDa (CaR), were detected. Controls included antibody pre-absorbed with a 10-fold excess of immunizing peptide (*data not shown*).

4. Results: *Activating the CaR alters the localisation of ECAD in MIN6 cells*

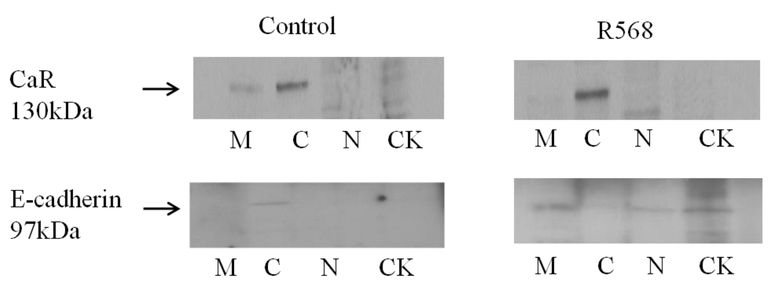


Figure 2: MIN6 cells were cultured in 5.5mM glucose for 48h with 0.5 mM Ca^{2+}± R568 (1µM). The expression level of E-cadherin and CaR were then determined by immunoblotting. Cell compartment analysis revealed re-localisation of the CaR from the membrane (M) into the cytosol (C) following R568 treatment. In contrast, R568 increased expression of E-cadherin throughout the cell with expression predominantly in the cytosol under control conditions, re-localising to the membrane (M), nuclear (N) and cytoskeleton (CK) fractions in response to the calcimimetic.

5. Results: *Activating the CaR increases ECAD expression in MIN6 cells.*

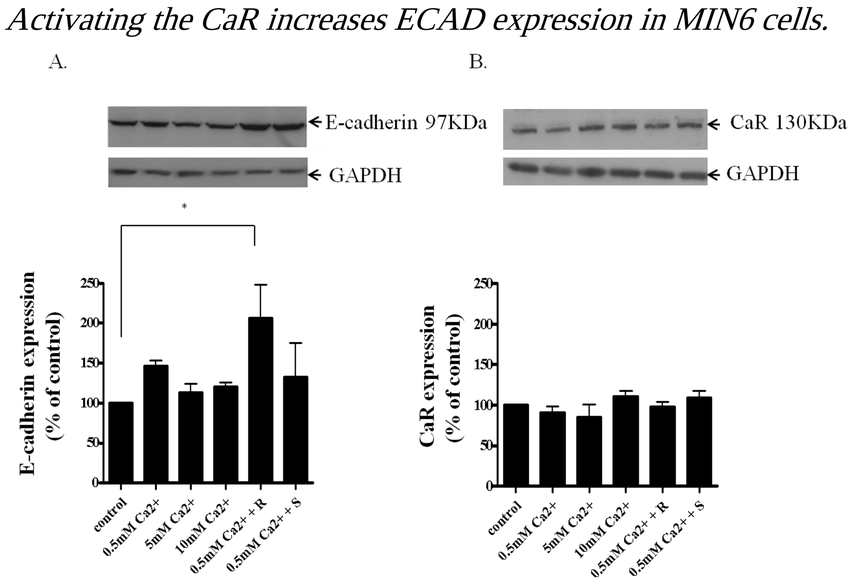
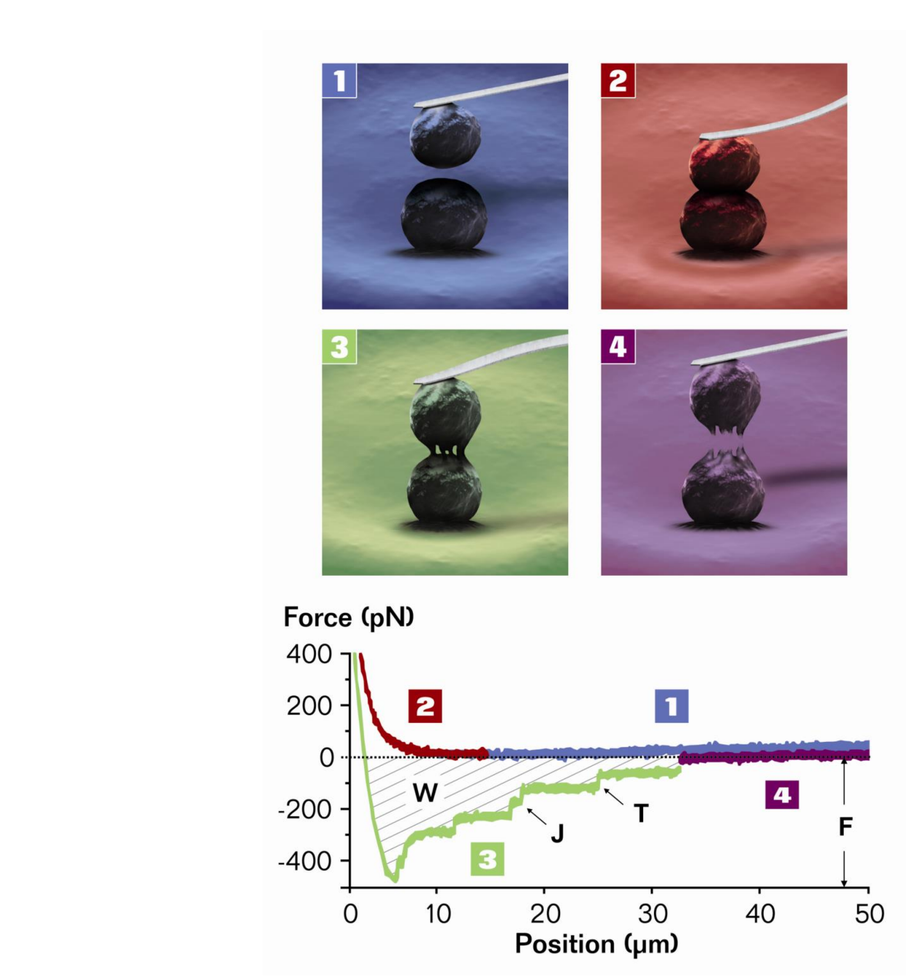


Figure 3: MIN6 cells were cultured in 5.5mM glucose for 48hr with 0.5,5,10mM Ca^{2+}± R568 or S568 (1µM). The expression level of (A) E-cadherin and (B) CaR were determined by immunoblotting. The upper panel shows representative immunoblots of E-cadherin and the CaR (upper blot) or the same blot stripped and re-probed for GAPDH as a loading control (lower blot). Band intensities were quantified by densitometry and the non-stimulated, nominal Ca^{2+} condition normalized to 100% and all other conditions compared to this. The lower panels shows the mean+sem. derived from densitometry of 3 blots. Each bar in the histogram represents the same condition in the blots above. Key significances are shown, **P*<0.05, n=3.

6. Results: *Activating the CaR increases functional coupling between MIN6 cells.*



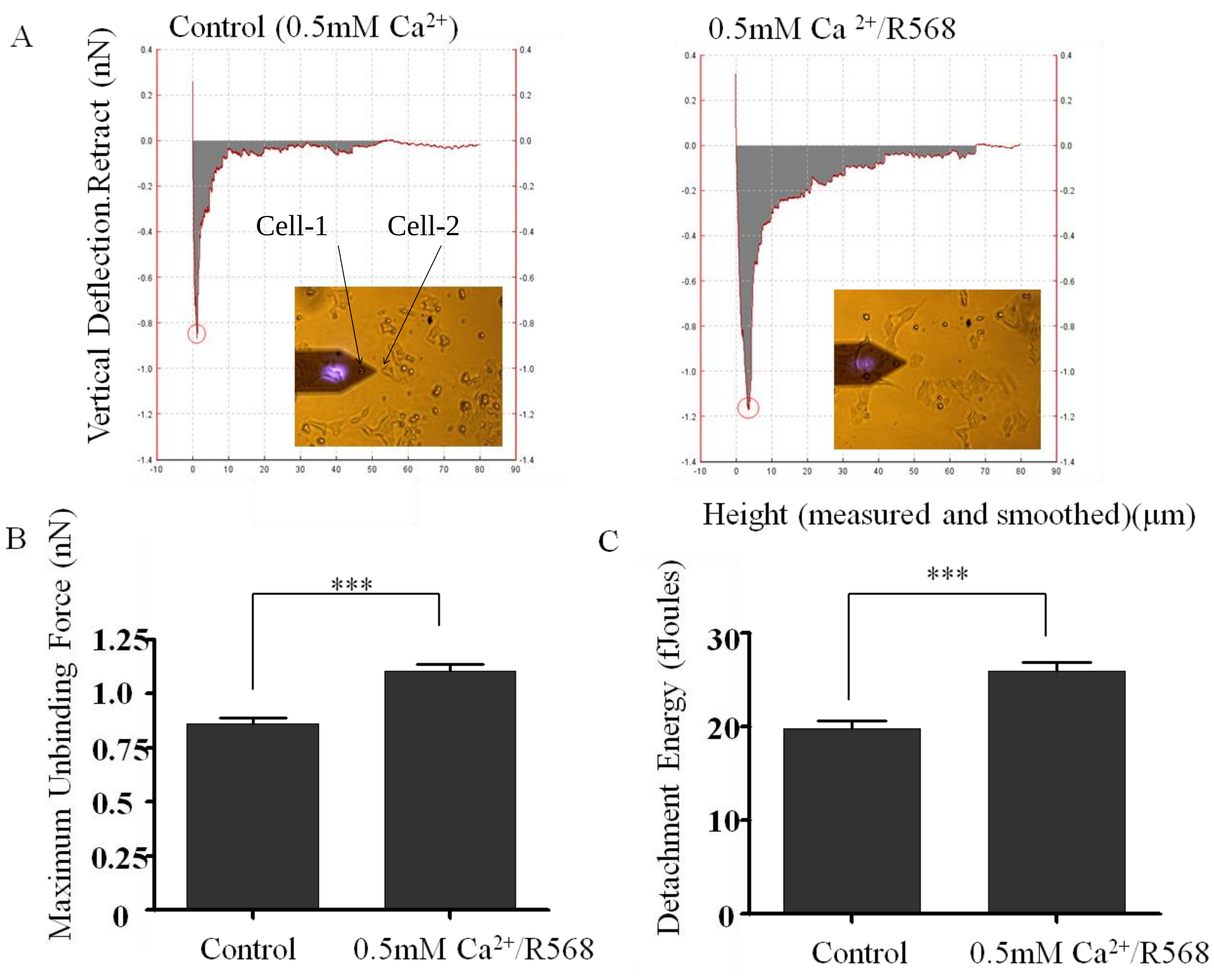


Figure 4: Single-cell force-spectroscopy was used to measure cell-to-cell adhesion and the separation forces required to uncouple cells. Prior to attachment, cells were cultured for in 5.5mM glucose for 48hrs under identical conditions +/-R568 (1µM). A single MIN6 cell (Cell-1) was bound to a cantilever and subsequently brought into contact with an adherent cell (Cell-2) within a cluster, using a fixed force (Fig4A phase inserts). After 10sec, the cantilever was then retracted (5µm/sec) and force versus displacement measured until the cells were completely separated. Retraction force-displacement curves 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 later the "maximum unbinding force" (panel B). The retraction measurements of control (41cells in 4 separate experiments) and R568 (1µM)-treated MIN6 cells (36cells in 4 separate experiments) are shown in the panels B and C. The results indicate that the calcimimetic R568 increases the maximum unbinding force by 29±3.1% (n=4; *P*<0.001), whilst the detachment energy was increased by 31±3.9% (n=4; *P*<0.001).

7. Conclusions:

a) Our data confirm that the CaR is expressed on MIN6 cells.
b) Activation of the CaR increases the expression of epithelial E-cadherin and alters it's cellular localisation .
c) Activation of the CaR significantly increases functional cell-to-cell adhesion between pancreatic beta-cells and increases the force of detachment required to separate coupled cells.
d) Our data provides a mechanism by which CaR activation may improve cell coupling and thus enhance insulin secretion from islets.

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