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A role for the extracellular calcium-sensing receptor in β-cell proliferation

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1. Background:

The extracellular calcium-sensing receptor (CaR) is a 7-transmembrane spanning G-protein-coupled receptor commonly associated with the regulation of systemic Ca^{2+}-homeostasis. However, it is becoming increasingly apparent that the CaR has diverse effects outside this primary role.

Numerous studies have now investigated the role of the CaR on proliferation. In the colon, activation of the calcium receptor up regulates adherens junction proteins, and a loss of CaR expression is associated with abnormal differentiation and progression of carcinomas [1].

In keratinocytes, inactivation of CaR expression has been shown to suppress the assembly of the cell adhesion associated epithelial-cadherin (ECAD) and β-catenin-PI3K complex [2]. Pancreatic β-cells express the CaR [3]. From our own studies, pancreatic β-cells demonstrate a close association between adherens junction proteins and secretory granules [4]. Neutralising ECAD-mediated cell adhesion decreases glucose-evoked synchronicity in Ca^{2+}-signals between adjacent cells within islets [5]. Together, these findings suggest an intriguing link between cell-cell adhesion, CaR expression and proliferation in pancreatic β-cells.

Elevated $[Ca^{2+}]_e$ can activate p38 and p42/44 MAPkinases [6, 7] and increase cell proliferation in pancreatic β-cells [8]. In the current study, we have examined whether specific activation of the CaR, using the calcimimetic R568, enhances β-cell proliferation at physiologically appropriate concentrations of extracellular calcium. The involvement of MAPK signalling cascade in transducing signals from the CaR was be determined using the MEK/ERK inhibitors PD098059 and SU1498.

Down-regulation of the CaR induces down-regulation of adheren junction proteins and reduces proliferative capacity in skin, colon and pancreatic β-cells. In the current study, we have assessed the effect of reduced CaR expression on proliferative capacity when the cells are grown as either 2-dimensional monolayers or 3-dimensional pseudoislets, to ascertain whether cell configuration and the degree of cell-cell contact, alters CaR expression and ultimately the β-cell turnover. Cell-cell adhesion was reduced further by immuno-neutralising ECAD in order to test the effect on CaR-expression and calcium-mediated proliferation.

2. Methods:

***Maintenance of MIN6 cells*:**
❑MIN6 pseudoislets (PI) were cultured under the same conditions as monolayers (M), in tissue culture flasks pre-coated with gelatin (1% w/v). MIN6 pseudoislets, formed after 5 days in gelatin-coated (1%) 6-well plates were incubated overnight in the presence of rat IgG or rat anti-E-cadherin (60µg/ml, Sigma, UK).

CaR expression in MIN6 cells:
❑Western blot analysis: Filters were analyzed with polyclonal rabbit anti-CaR serum (1:1000) raised against a synthetic peptide corresponding to residues 18–29 of the mouse CaR (CSAYGPDQRAQKK; Genosphere Biotechnologies, Paris, France). Membranes were incubated with the secondary antibody (horseradish peroxidase-conjugated) anti-rabbit (1:3000).

❑For determination of protein localisation within the cell, proteins were harvested using the Qproteome cell compartment kit.

❑CaR immunoreactivity was assessed by fluorescence immunocytochemistry using a polyclonal rabbit anti-CaR serum (1:1000, as above), and ALEXA 488 or 594-conjugated chicken anti-rabbit IgG (Molecular Probes 1:2000, 1h).

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. Cells were serum starved overnight in DMEM containing glucose (5mM) and low calcium (0.5mM). As required, the MEK inhibitor PD98059 or SU1498 was applied as a 1hr pre-treatment, before adding the incubation media containing; calcium (0-10mM), the calcimimetic (+/-R568 (1µM) Amgen Inc,Thousand Oaks, CA, USA) and +/-PD98059 or SU1498 (1-100µM). All solutions contained BrdU (10µM) for the final incubation. Proliferating cells were visualised using Alexa-594-conjugated anti-BrdU (Molecular Probes, Invitrogen) at 1:200.

Data Analysis:
❑Autoradiographs were quantified by densitometry using TotalLab 2003 (NonLinear Dynamics, Durham, NC USA). Controls were 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 where P<0.05 was considered significant.

3. Results:

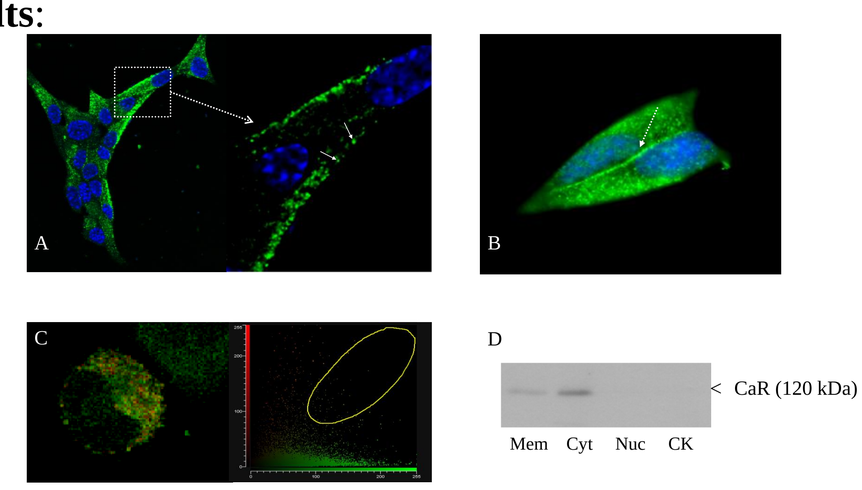

Figure1. *The CaR is expressed in insulin-immunoreactive cells:* Insulin-immunoreactivity (green) and nuclear staining (visualised with DAPI, blue) in MIN6 cells is shown in panel (A). CaR expression (visualised using green ALEXA 488 (B) demonstrates punctate cytosolic staining in addition to clear expression at the interface between adjacent cells. Western blot analysis (D) confirmed that CaR expression was maximally expressed in the cytosolic and membrane fractions of MIN6 cells. Similar cytosolic localisation for the CaR protein (red) is observed in isolated insulin-immunoreactive cells (green) from primary mouse pancreata (C). The extent of co-localisation between insulin-immunoreactivity and CaR-expression was determined using Image J software (freeware obtainable from the National Institutes of Health Web site). The degree of co-illuminated pixels (yellow oval) was negligible, indicating that the CaR is not co-localised on the secretory granules.

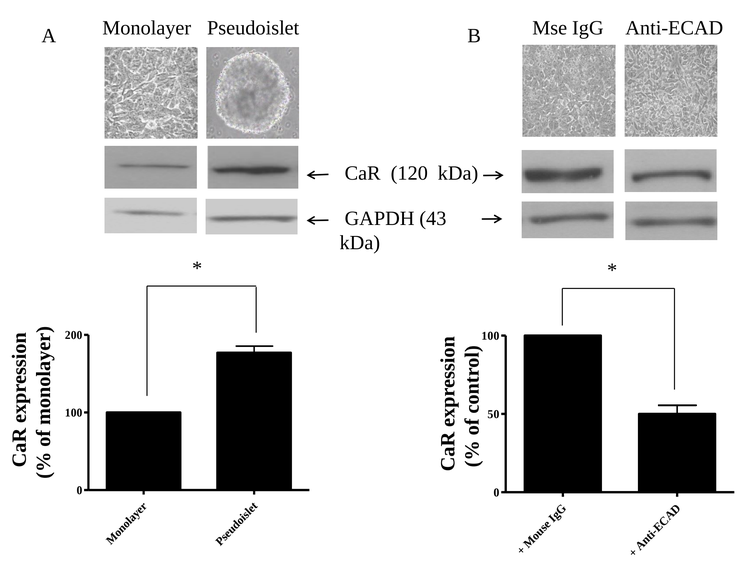

Figure2. *Cell architecture affects CaR-expression:* (A) Western blotting analysis confirming the expression of the CaR (120kDa) and GAPDH (43kDa) from 3 separate preparations of MIN6 cell lysates (50µg), demonstrated enhanced CaR expression (75%) in PIs compared to their monolayer counterparts ($P<0.05$, n=3). Conversely, the effect of an overnight incubation with (68µg/ml) immuno-neutralizing anti-ECAD (B) halves CaR-expression compared to IgG-treated control cells. Note the gross change in cell morphology and the reduction in close cell-to-cell contact following the antibody treatment Key significances are shown, *$P<0.05$, n=3.

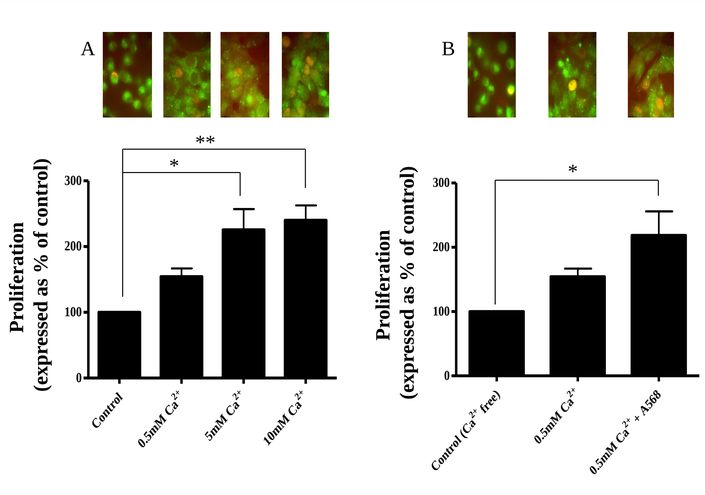

Figure3. *Stimulation of the CaR increases cell proliferation in MIN6 cells:* Cell proliferation was determined by the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into MIN6 DNA and visualized by ALEXA 594-tagged anti-BrdU (red). All data were recorded in the absence of FCS and expressed as a % of Ca^{2+}-free control. (A) Increasing extracellular calcium ($[Ca^{2+}]e$, 0.5-10mM) evoked a concentration-dependent increase in the % of proliferative cells in monolayer cultures. (B) Small increases in proliferation induced by low $[Ca^{2+}]e$ (0.5mM) were significantly enhanced in the presence of the calcimimetic R568. Data represent mean ± SEM where * denotes $P<0.05$ and ** $P<0.01$; n=3 for each.

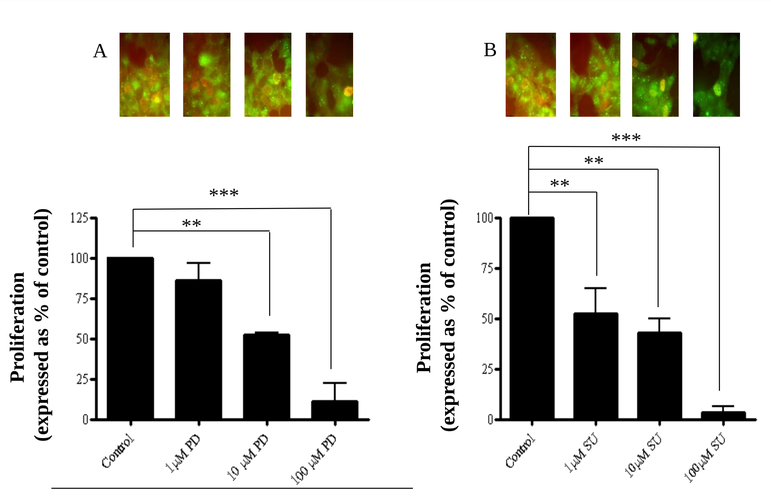

Figure4. *The effect inhibiting the MAP kinase pathway on BrdU incorporation into MIN6 β-cell DNA*: MIN6 β-cells were seeded onto APES-coated coverslips (50,000 cells/well) and the cells allowed to adhere overnight. The cells were incubated for a further 24hr in the presence of low extracellular calcium (0.5mM). The inhibitors, were present for the final 3hrs of the incubation and BrdU (10µM) was added for the final 2hrs. Fluorescence microscopy was used to detect BrdU incorporation using an ALEXA 594-tagged anti-BrdU antibody. (A) Inhibition of the p42/44 MAPK pathway using the MEK inhibitor PD098059 (1-100µM) significantly reduced proliferation in a concentration-dependent manner. (B) Similar inhibition was obtained using SU1498 (1-100µM) which inhibits ERK kinase activity. Key significances are shown, *$P<0.05$, ** $P<0.01$, *** $P< 0.001$, n=3.

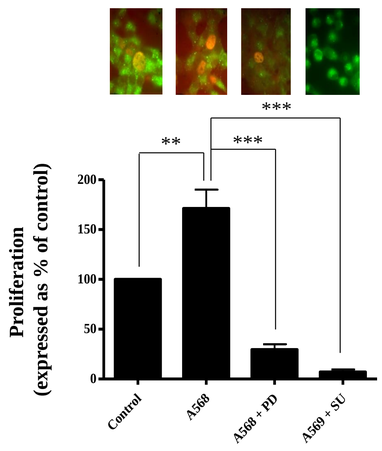

Figure5. *CaR-evoked proliferation is dependent on MAPK signalling:* Activation of the CaR using the Calcimimetic R568 (1µM) at low extracellular Ca^{2+} (0.5mM) significantly increased the % of cells undergoing proliferation ($P<0.01$, n=3). This CaR mediated increase in cell turnover was inhibited by both PD098059 (71%) and SU1498 (93%). Key significances are shown, *$P<0.05$, ** $P<0.01$, *** $P< 0.00$, n=3.

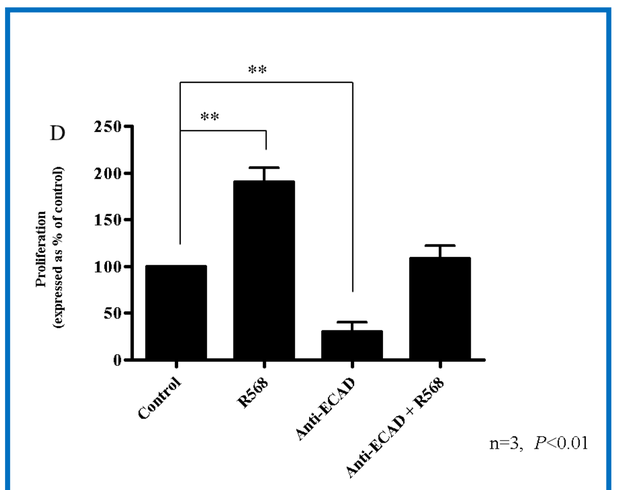

Figure6. *Loss of cell-to-cell contact reduces CaR-mediated proliferation:* Overnight incubation with (68µg/ml) immuno-neutralizing anti-ECAD decreases cell-cell contact and significantly reduces the ability of the calcimimetic R568 (1µM) to increase BrdU incorporation compared to IgG-treated control cells. Key significances are shown, *$P<0.05$, n=3.

4. Summmary:

❑We have previously shown that activation of the CaR in pancreatic β-cells can increase insulin secretion. These studies confirmed that the CaR has an important role mediating cell-to-cell communication within islets to co-ordinate insulin secretory responses.

❑In the present study we have confirmed that activation of the CaR, via either elevated extracellular calcium or co-administration of the calcimimetic R568, increases β-cell turnover.

❑The proliferative effects of CaR appear dependent on whether the cells are grown as monolayers or pseudoislets.

❑Down-regulation of CaR expression was observed in cells treated to neutralise E-cadherin ligation to reduce cell-cell contact when. Under these conditions, CaR-stimulated proliferation was reduced by X% as compared to control.

❑These data suggest that close cell-to-cell contact directly influences CaR signalling and implicates other molecules such as the adherens junction proteins.

Conclusions:

Defining factors/mechanisms that regulate proliferation of β-cells is of considerable importance in understanding the maintenance of β-cell mass *in vivo*. Furthermore, this information may ultimately be used to isolate and expand β-cells for therapeutic use.

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