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E-Cadherin and Cell Adhesion: a Role in Architecture and Function in the Pancreatic Islet

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Key Words

E-cadherin (ECAD) • Cell-to-cell communication • Pancreatic islets • Insulin

Abstract

Background/Aims: The efficient secretion of insulin from β-cells requires extensive intra-islet communication. The cell surface adhesion protein epithelial (E)-cadherin (ECAD) establishes and maintains epithelial tissues such as the islets of Langerhans. In this study, the role of ECAD in regulating insulin secretion from pseudoislets was investigated. Methods: The effect of an immuno-neutralising ECAD on gross morphology, cytosolic calcium signalling, direct cell-to-cell communication and insulin secretion was assessed by fura-2 microfluorimetry, Lucifer Yellow dye injection and insulin ELISA in an insulin-secreting model system. Results: Antibody blockade of ECAD reduces glucose-evoked changes in $[Ca^{2+}]_i$ and insulin secretion. Neutralisation of ECAD causes a breakdown in the glucose-stimulated synchronicity of calcium oscillations between discrete regions within the pseudoislet, and the transfer of dye from an individual cell within a cell cluster is attenuated in the absence of ECAD ligation, demonstrating that gap junction communication is disrupted. The functional consequence of neutralising ECAD is a significant reduction in insulin secretion. Conclusion: Cell adhesion via ECAD has distinct roles in the regulation of intercellular communication between β-cells within islets, with potential repercussions for insulin secretion.

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Introduction

Increases in plasma glucose concentration initiate changes in cytosolic calcium and insulin secretion from pancreatic β-cells. However, individual β-cells show heterogeneity in their sensory, biosynthetic and secretory capabilities in response to nutrient stimulation [1-3]. Thus the functional unit of insulin secretion is the islet where synchronous glucose-induced oscillations of cytosolic calcium ($[Ca^{2+}]_i$) are required for efficient insulin release. Several theories have been proposed to explain the synchronous and cooperative activity of islets when compared to independent events observed in isolated individual β-cells including; the presence of other endocrine cells in islets [4], increased concentration of

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a diffusible transmitter in islets [5, 6] and activity of gap junctions [7]. Recent reports have also suggested that cell adhesion molecules, such as epithelial (E)-cadherin (ECAD), may also have a role in maintaining islet architecture and regulating insulin secretion [8, 9].

ECAD is a transmembrane protein whose extracellular domain forms calcium-dependent homodimers with cadherins expressed on neighbouring cells facilitating cell-to-cell adhesion [reviewed in 10]. The extracellular domain is linked to the intracellular domain by a hydrophobic membrane-spanning region. The cytoplasmic domain of the cadherins binds to the α- and β-catenins linking the cadherins to the actin cytoskeleton. 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 [11, 12]. In developing mouse pancreas, ECAD plays an essential role in allowing β-cells to cluster in to islet structures [13]. Over-expression of dominant negative hepatocyte nuclear factor-1 in mouse β-cells (the gene responsible for type 3 MODY) results in abnormal islet architecture, down-regulation of E-cadherin and reduced insulin secretion [14, 15]. Similarly, the MIN6 cell line sub-clone (C3) has been identified with reduced glucose evoked insulin secretion and down-regulated E-cadherin expression [16], whilst in rat pancreatic islets, increased expression of the adherens proteins α- and β-catenin is correlated with increased glucose evoked insulin secretion [17]. It is clear that E-cadherin not only plays an important role in islet development but also in the continued function of the islet in terms of glucose responsiveness and insulin secretion. However, exactly how E-cadherin and the other adherens junction proteins control glucose evoked insulin secretion remains unclear. In the present study we assess the effect of immuno-neutralising ECAD on gap-junction-mediated cell-to-cell communication and insulin secretion.

Materials and Methods

Maintenance of MIN6 cells and formation of pseudoislets

MIN6 cells 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. MIN6 pseudoislets were cultured under the same conditions as monolayers, in tissue culture flasks pre-coated with gelatin (1% w/v) [1]. MIN6 pseudoislets, formed after 5 days in gelatin-coated (1%) 96-well plates were incubated overnight in the presence of rat IgG (Sigma, UK; final concentration, 68μg/ml) or rat anti-E-cadherin (Sigma UK product code U3254 – anti-uvomorulin, raised against a mouse immunogen; final concentration, 68μg/ml). Effects upon pseudoislet morphology were assessed by light microscopy.

Calcium Microfluorimetry

Pseudoislets were transferred to APES-coated coverslips and maintained in DMEM containing either rat IgG or rat anti-E-cadherin overnight. Cells were loaded for 30 minutes at 37°C with 5μM 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 $CaCl_2$, 0.8mM $MgSO_4$, 0.3mM Na_2HPO_4, 0.4mM KH_2PO_4, 4.2mM $NaHCO_3$, 10mM HEPES and 2mM glucose, pH 7.4). A low-pressure rapid-superfusion system (flow rate 1-2ml/min) was used to change the solutions in the bath. 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).

Dye transfer experiments

As previously described [18], an individual cell within a cell cluster was microinjected with Lucifer yellow CH, dilithium salt (Sigma), dissolved in 250μl of fresh 150mmol/L LiCl/10mmol/L Hepes (pH 7.2). Briefly, cells were microinjected using femtotips (0.5±0.2μm internal diameter) and the Injectman/Femtojet 5247 delivery system (Eppendorf, Hamburg, Germany), for a duration of 3 seconds (injection pressure of 252hPa, compensation pressure of 114hPa). Dye transfer was recorded over a 3-minute period using Metamorph acquisition software (Universal Imaging Corp Ltd, Marlow, Bucks UK).

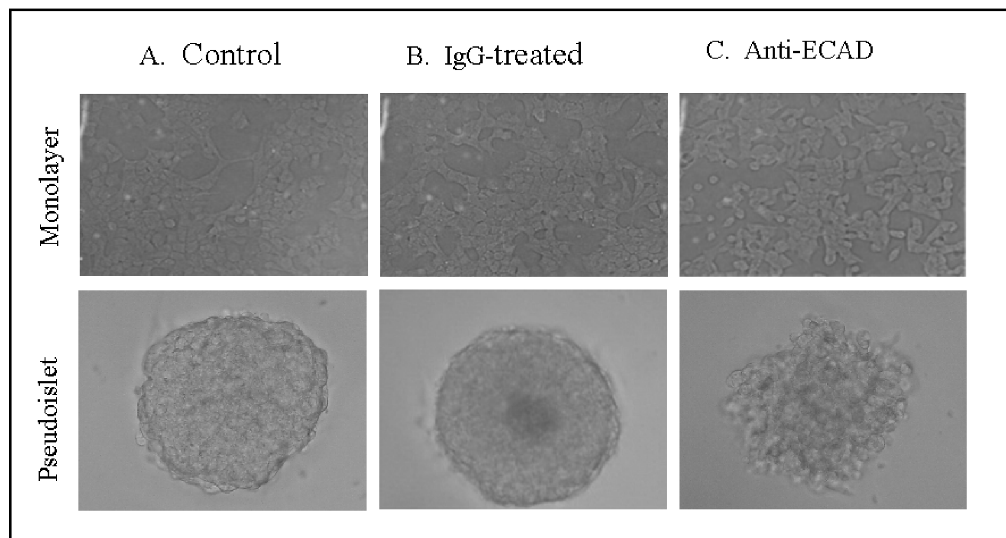
Insulin secretion

Eight pseudoislets per well were cultured overnight in DMEM containing either rat IgG or rat anti-E-cadherin antibodies. Pseudoislets were pre-incubated in a bicarbonate-buffered physiological salt solution containing 2mM glucose, 2mM $CaCl_2$ and 0.5mg/ml BSA for 2 hours [19]. The cells were incubated for 1 hour in the salt solution to establish secretion under basal conditions followed by 1 hour in the same salt solution supplemented with secretagogues. The insulin content of the incubation medium was assessed by ELISA (Immunodiagnostic Systems Ltd.).

Data Analysis

Secretion and microfluorimetry data are expressed as mean±SEM. Differences in secretion were assessed by the Mann-Whitney U-test and calcium data was assessed via unpaired students *t*-test. A probability (*P*) value <0.05 was considered statistically significant.

Fig. 1. Immuno-neutralization of endogenous E-cadherin alters morphology of MIN6 pseudoislets: Panel A demonstrates a phase image of MIN6 monolayer and pseudoislets incubated with rat IgG (68µg/ml). The effect of an overnight incubation with (68µg/ml) immuno-neutralizing anti-ECAD is shown in panel B. Note the gross change in cell and islet morphology and the reduction in close cell-to-cell contact following the antibody treatment.



Results

Effect of E-cadherin antibody on pseudoislet structure

Figure 1 shows images of MIN6 cells when grown in monolayer cultures (upper panels) or as cell clusters, termed pseudoislets, following culture on gelatin-coated plastic (lower panel). Figure 1A shows that under control conditions, pseudoislets form tightly packed three-dimensional structures containing between 3000-5000 cells [1]. Incubation of preformed pseudoislets with a monoclonal antibody directed against the extracellular domain of E-cadherin dramatically altered the overall architecture, producing 'fluffy' loosely aggregated cell clusters (Figure 1C lower panel). These gross morphological changes were not observed when pseudoislets were incubated with a non-specific IgG (Figure 1B lower panel). Treatment of MIN6 cells, configured as monolayers or pseudodislets, with either IgG or anti E-CAD antibody, had no effect on cell viability (when compared to control), with greater than 99% of cells excluding trypan blue vital stain in each case (data not shown). We have recently shown that this anti-E-cadherin antibody recognises endogenous E-cadherin (120kDa) in MIN6 cells by both western blotting and following imunoprecipitation of the E-cadherin interacting protein β-catenin [9]. Pseudoislets were unable to form when ECAD was neutralised during the normal 5-day culture period of the islets (results not shown). Thus, E-cadherin is required to both establish and maintain pseudoislet structure, data consistent with previous studies [8].

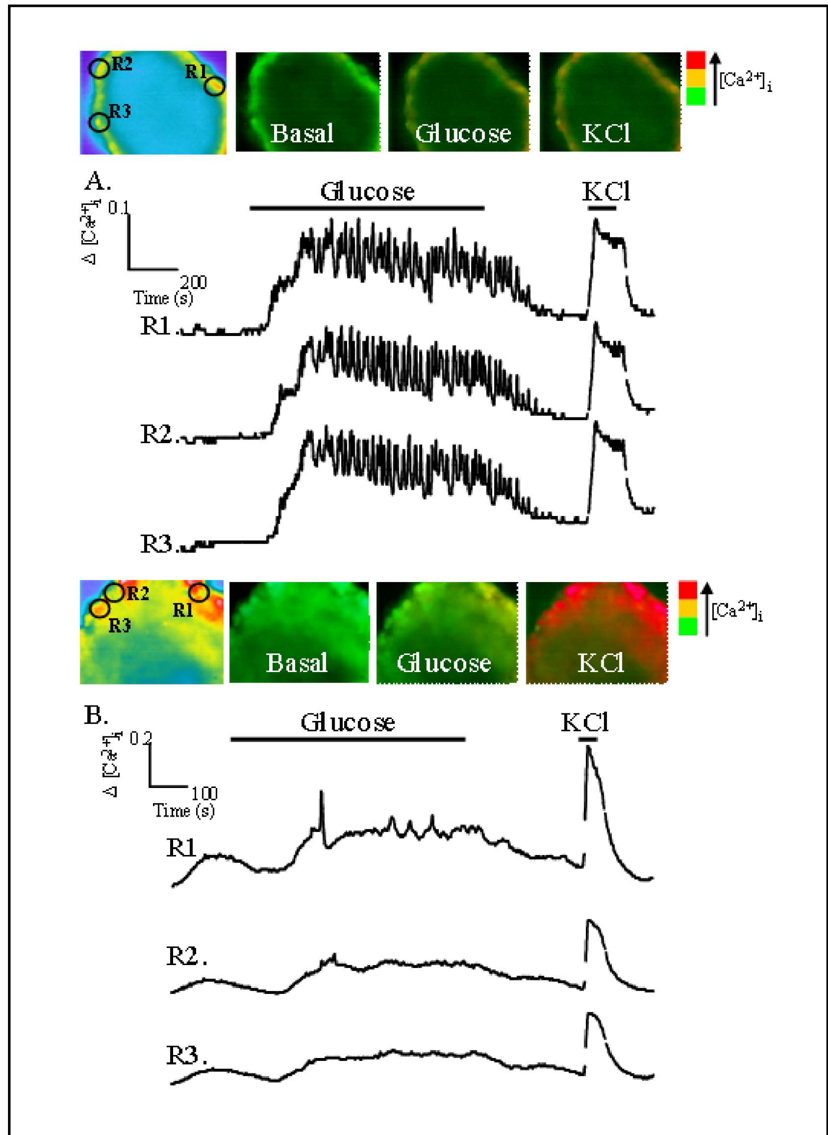
Cadherin-activated signalling within pseudoislets effects nutrient-evoked $[Ca^{2+}]_i$ oscillations

Not only is E-cadherin important as an adhesion molecule but it might also have effects upon β-cell function [8]. To investigate this, nutrient (20mM glucose)-evoked changes in cytosolic calcium were assessed in pseudoislets treated with either anti-E-cadherin antibody or non-specific IgG. It should be noted that the calcium-responsive dye Fura-2am penetrated more deeply in to the core of immunoneutralised pseudoislets (Figure 2B-'basal') when compared to control (IgG treated) islets (Figure 2A-'basal') thereby demonstrating the altered islet morphology and cell-to-cell contact that is induced by neutralisation of E-cadherin.

Figure 2A shows that in IgG treated pseudoislets, glucose evoked synchronous and propagating calcium oscillations. The response declined rapidly following removal of the stimulus (37/37 regions examined in four separate experiments). These results are identical to those obtained for untreated pseudoislets (results not shown). Figure 2B shows that pseudoislets treated with anti-E-cadherin remained glucose-responsive, exhibiting a sustained glucose-evoked rise in $[Ca^{2+}]_i$ upon stimulation (Figure 2B; 30/40 regions in four separate experiments). However, the change in $[Ca^{2+}]_i$ lacked significant oscillations above the mean elevated plateau and small $[Ca^{2+}]_i$ transients failed to propagate into adjacent regions of the islet (Figure 2B).

Quantification of the effects of ECAD-neutralisation on nutrient-evoked changes in $[Ca^{2+}]_i$ in islets showed that the basal ratiometric estimates of cytosolic calcium (340/380nm) were comparable between pseudoislets pre-

Fig. 2. Effect of glucose on Ca^{2+}-signaling MIN6 pseudoislets following immuno-neutralization of endogenous E-cadherin: Application of glucose (20mM) evoked a sustained rise in $[Ca^{2+}]_i$ in MIN6 PIs treated with both rat IgG (panel A) or anti-ECAD immuno-neutralizing antibody (panel B). Although both islets retain glucose-responsiveness, inhibition of ECAD ligation reduces the frequency of Ca^{2+}-transients and prevents the transfer of oscillatory changes between discrete regions within a single islet.



treated with IgG- or anti-E-cadherin (IgG-treated: 0.95±0.04, 38 observations, n=4; anti-E-cadherin-treated: 0.95±0.04, 40 observations, n=4; Figure 2). This data clearly shows that resting levels of calcium in anti-ECAD treated and control islets are comparable and that the normal homeostatic mechanisms that control intracellular calcium levels are intact. To quantify the change in amplitude of agonist-induced increases in $[Ca^{2+}]_i$, basal-to-peak levels were normalised to the KCl (20mM)-evoked increases in cytosolic calcium and expressed as a % (see table 1). Table 1 shows that the highest peak amplitude of glucose-evoked $[Ca^{2+}]_i$ reached 73% of the KCl-evoked response following IgG-treatment. The anti-ECAD antibody reduced the basal-to-peak amplitude of the nutrient-evoked change in $[Ca^{2+}]_i$ to 43% of the KCl response (see table 1).

	IgG treated pseudoislets	Anti-ECAD treated pseudoislets	Statistical analysis (students T test)
Glucose	73 ±5	43±4	P<0.0001
Tolbutamide	80±4	51±2	P<0.0001
ATP	105±5	87±4	P<0.001

Table 1. Nutrient and non-nutrient evoked changes in basal-to-peak Ca^{2+} response (expressed as a percentage of KCl response): Basal to peak calcium transients were monitored as changes in the ratio of 340/380nm excitation by fluorescent microscopy in Fura2-loaded pseudoislets treated with anti ECAD or IgG. At least 9 regions of interest were monitored simultaneously for stimulated changes in $[Ca^{2+}]_i$. Data is from 3 separate experiments (minimum 27 separate determinations, maximum 38 determinations) in each condition and is expressed a percentage (%) of the KCl evoked basal-to-peak change in calcium. Statistical significance was determined using the Students T-test.

Cadherin-activated signalling within pseudoislets effects non-nutrient-evoked $[Ca^{2+}]_i$ oscillations

Efficient insulin secretion depends on an appropriate response to a rise in plasma glucose. This response is often modulated by numerous potentiators that act to augment secretion. Activation of G-protein coupled receptors, such as the P_2-purinoreceptors, can have important modulatory effects on β-cell function [20], 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 assess the effect of neutralising E-cadherin on these important receptor-mediated and non-nutrient evoked changes in $[Ca^{2+}]_i$ we examined the effects of ATP (50μM), Tolbutamide (10μM) and KCl (20mM) on changes in $[Ca^{2+}]_i$ from pseudoislets cultured in either IgG (Figure 3A) or anti-E-cadherin antibody (Figure 3B).

Figure 3 shows that ATP, tolbutamide and KCl evoked increases in intracellular calcium in both anti-E-cadherin and IgG treated pseudoislets (27/27 from regions in IgG-treated pseudoislets and 30/30 regions in pseudoislets treated with anti-E-cadherin in a total of six separate experiments). However, neutralisation of cadherin activity reduced significantly the basal-to-peak amplitude of both ATP and tolbutamide-evoked changes in $[Ca^{2+}]_i$ in comparison to KCl-evoked changes (see table 1). Thus, the basal-to-peak $[Ca^{2+}]_i$ change induced by tolbutamide was reduced from 80% of the KCl response in IgG treated pseudoislets to 50% in pseudoislets treated with anti-ECAD ($p<0.0001$). Neutralisation of ECAD caused an 18% reduction in ATP-induced changes in basal-to-peak $[Ca^{2+}]_i$ (normalised to the KCl response) that was a statistically significant effect ($p<0.01$) when compared to non-specific IgG treatment. In addition, Figure 3B shows that in IgG treated pseudoislets, ATP induced a biphasic change in $[Ca^{2+}]_i$ that consisted of a rapidly rising phase initiated by addition of ATP, followed by a slower and oscillatory falling phase, with a significant number of $[Ca^{2+}]_i$ oscillations continuing after removal of stimulating ATP. In contrast, in ECAD-neutralised pseudoislets, ATP induced a single rapidly rising and falling $[Ca^{2+}]_i$ oscillation with no extended second phase and no continuing oscillations (Figure 3B).

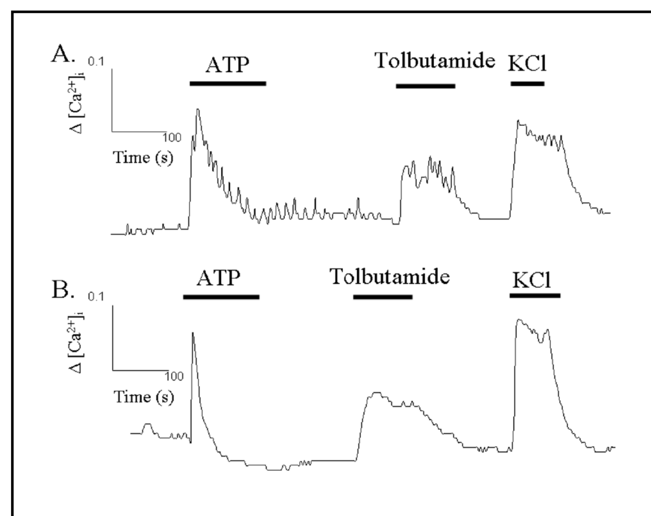


Fig. 3. Effect of non-nutrients on Ca^{2+}-signaling MIN6 pseudoislets following immuno-neutralization of endogenous E-cadherin: ATP (50μM), tolbutamide (10μM) and KCl (20mM)-evoked changes in $[Ca^{2+}]_i$ in MIN6 PIs treated with either rat IgG (panel A) or anti-ECAD immuno-neutralizing antibody (panel B). Responses are of comparable basal-to-peak amplitude, with the only noticeable difference being a loss in the sustained Ca^{2+}-influx phase of the purinergic response to ATP.

E-cadherin ligation regulates gap junction communication

Several studies have shown that E-cadherin-activated signalling is required for gap junction formation and can affect cell communication between coupled cells [21-24]. To assess the effect of disrupting E-cadherin on direct cell-to-cell communication we examined the effect of immuno-neutralising E-cadherin on transfer of the membrane impermeant dye Lucifer Yellow. Figure 4A shows that Lucifer Yellow spreads throughout untreated cell clusters monitored during a 3-minute period following microinjection of the dye in to a single cell within a tightly associated cluster. Similarly, treatment of the cells with non-specific IgG does not affect dye transfer within MIN6 cell clusters (Figure 4B). In control and IgG treated pseudoislets dye was seen to transfer to 3-4 neighbouring cells within 3 minutes in 4 separate experiments. However, anti-E-cadherin treatment of pseudoislets prevented dye spread from the injected cell into any neighbouring cells after 3min (4 separate experiments) and after 10 minutes (results not shown). Similarly, incubation of cell clusters with heptanol, a known uncoupler of gap-junctions also prevented dye transfer within 3 and 10 minutes. Data presented in figure 4 is one experiment representative of 4.

Immunoneutralization of E-cadherin attenuates glucose-evoked insulin secretion

Immuno-neutralisation of E-cadherin alters pseudoislet morphology, disrupts Ca^{2+}-signalling and inhibits direct cell-to-cell communication (Figures 1-4). To assess the functional consequence of these changes

Fig. 4. Loss of direct cell-to-cell communication in MIN6 cells following immuno-neutralisation of endogenous E-cadherin: The transfer of Lucifer Yellow suggests direct cell-to-cell communication between coupled MIN6-cells. The monochrome plates illustrate phase images of MIN6-cell clusters. The fluorescence image of the same cell clusters following microinjection of Lucifer Yellow at time 0min. The same field of view is recorded three minutes after single-cell injection of the dye in control cells (4A), in cells incubated with rat IgG antibody (68µg/ml; 4B), in the presence of heptanol (2.5mmol/L; 4C), and in cells incubated with the E-cadherin immuno-neutraling antibody (68µg/ml; 4D). Data presented 1 experiment representative of 4.

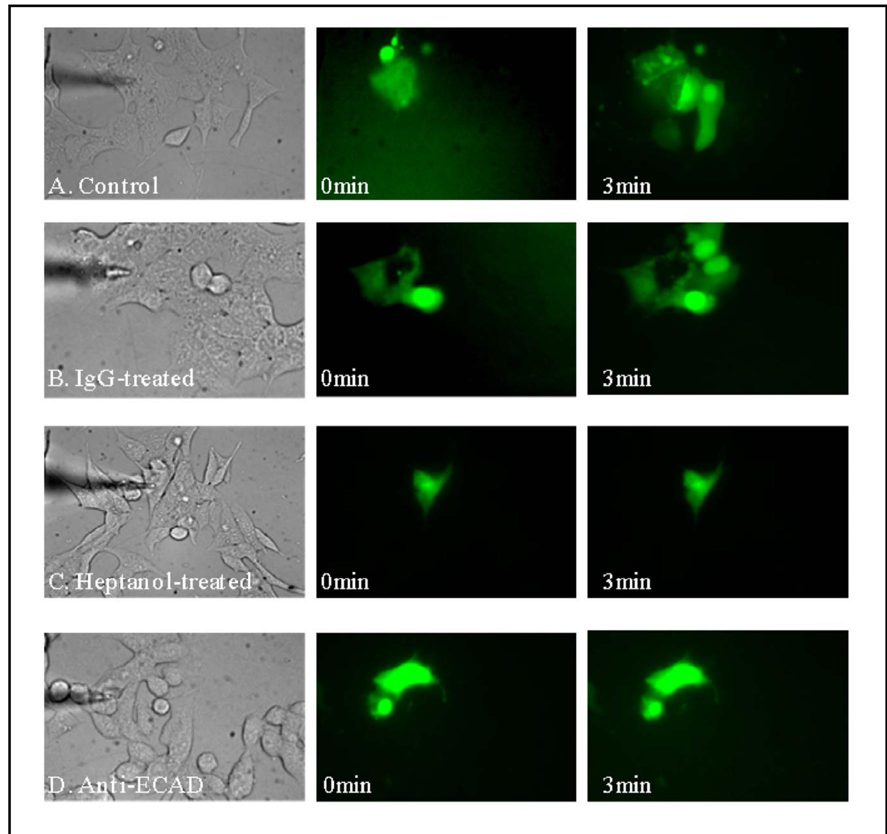
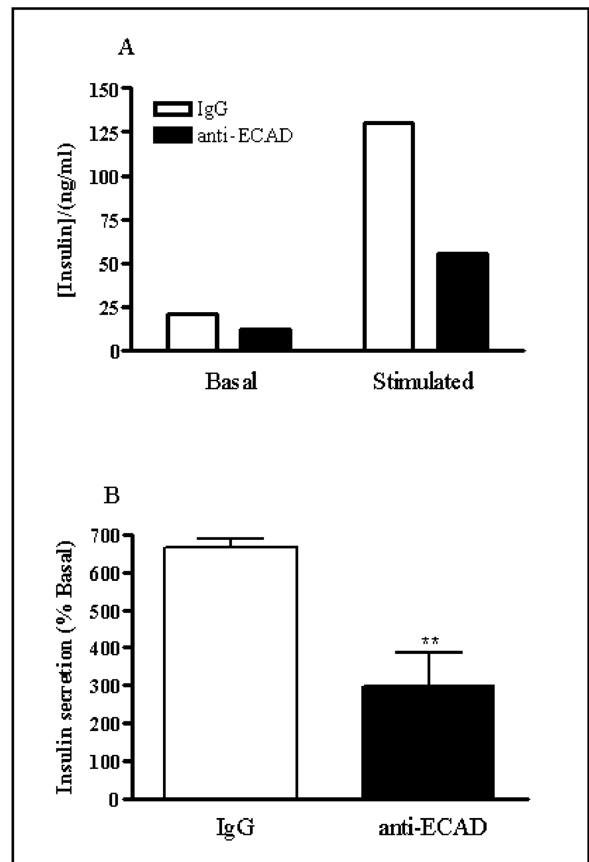


Fig. 5. The effect of neutralizing E-cadherin on insulin secretion from MIN6 pseudoislets: Pseudoislets cultured for 6 days were incubated with either rat IgG (control) or an immuno-neutralising E-cadherin antibody for a further 24hrs. Insulin release (quantified in ng/ml) in response to stimulation with 20mM glucose was measured by ELISA, with Panel A showing results from a single representative experiment. Panel B shows combined data from 4 separate experiments where insulin release is expressed as a percentage of basal secretion. Data are the mean ± SEM of four separate experiments, where ** signifies P<0.01. Abolishing E-cadherin cell contacts within the pseudoislet structure significantly inhibits glucose-evoked insulin secretion.



we examined the effect of reduced E-cadherin ligation on glucose-evoked insulin secretion. Insulin release was quantified from IgG or the anti-E-cadherin treated pseudoislets pre-incubated with low glucose (2mM), and then stimulated with high glucose (20mM). In figure 5, panel A, data from a single representative experiment demonstrates that E-cadherin immuno-neutralisation reduced both basal and glucose-stimulated insulin secretion. Although the effect on basal secretion was small, mean data from 4 separate experiments confirmed that the effect was significant (IgG; 18.2±1ng/ml, n=4

compared to anti-ECAD; 11.8±1.4ng/ml, n=4; $P<0.01$). However, the major effect of E-cadherin immuno-neutralisation was to reduce the magnitude of the glucose-evoked release of insulin. This is clearly shown in figure 5, panel B, which combines data from 4 separate experiments and expresses insulin secretion as % stimulated above basal. Thus IgG-treated islets exhibited a 6-7-fold increase in insulin secretion in response to glucose (665±24%, n=4), whereas pseudoislets treated with anti-E-cadherin showed only a 3-fold increase in insulin release (297±89%, n=4; $P<0.01$).

Discussion

The role of E-cadherin in regulating islet morphology, calcium homeostasis and exocytosis in MIN6 pseudoislets, was examined in a model system of insulin-secretion. Immuno-neutralising E-cadherin, showed that a loss in cell adhesion, reduces the number of glucose-evoked calcium transients and ablates their synchronicity between adjacent regions of the islet thereby reducing insulin secretion, and highlighting a central role for E-cadherin in islet structure and function.

E-cadherin has been shown to be up-regulated in MIN6 cells in psuedoislets compared to when grown in monolayers [1]. Ligation of ECAD and the subsequent activation of downstream signalling pathways, contributes to improved secretory efficiency of β-cells configured as an islet resulting from enhanced cell-to-cell contact [1, 25]. In the present study, antibody neutralisation of E-cadherin prevents the direct cell-to-cell transfer of the dye Lucifer Yellow in a manner similar to that observed when using the known gap-junctional uncoupler heptanol [26]. These findings are consistent with studies suggesting cadherin-mediated cell-cell adhesion is closely related to gap junction assembly/function in mouse epithelia [21-23] and rat cardiac myocytes [24]. Loss of gap-junction communication may therefore explain the reduced synchronicity in Ca^{2+}-signals between discrete regions of the pseudoislet.

In neuronal adherens junctions, it has recently been shown that catenins promote spatial organisation of presynaptic vesicles [27], and in pseudoislets we recently reported co-localisation between insulin containing vesicles and β-catenin [9]. Thus, the adherens junction and associated proteins play an important role in the organisation and function of neuro-endocrine tissues.

In the present study, although glucose evoked a rise in $[Ca^{2+}]_i$ the response lacked superimposed Ca^{2+}-oscillations. Similarly, the sustained oscillatory component of the receptor-mediated ATP-evoked rise in $[Ca^{2+}]_i$ was also lost following neutralisation of E-cadherin ligation. It is well known that stimulation of cells expressing P_{2Y} receptors with ATP results in a biphasic change in $[Ca^{2+}]_i$ that consists of $InsP_3$-dependent mobilisation of calcium from intracellular stores together with opening of plasma membrane channels facilitating entry of extracellular calcium [20]. Together these data demonstrate that E-cadherin may be involved in regulation of Ca^{2+}-entry through controlling the opening of plasma membrane Ca^{2+}-channels. It has been shown in neuronal cells, that N-cadherin regulates the opening of voltage-dependent calcium channels via the small G-proteins Rho and Rac-1 and the small G-protein activated kinase ROCK1 [28]. Alternatively, direct coupling via the cytoskeleton may provide mechanisms that link ECAD ligation to modulation of Ca^{2+}-homeostasis since it has been shown that force applied to N-cadherin is transmitted through the cytoskeleton to opening of the plasma membrane calcium channels [29].

Therefore, the adherens junction may be acting as a node for transmission of signals (activation of small G-proteins and PI3-kinase etc) that emanate from cell-to-cell contact and target the coupling of stimuli to secretion via regulation of the key Ca^{2+}-channels. Additionally these same signals have recently been proposed to regulate localisation of connexin-43 in cardiac myocytes [24]. Whether small G-proteins participate in these signals through their well-known roles in modulating the dynamics of F-actin structures or through initiating downstream signalling cascades (i.e. kinases) remains to be elucidated. From the current study, we propose that signals emanating from the adherens junction regulate islet structure, calcium entry dynamics and the function of gap junctions in β-cells, ultimately modifying glucose-evoked insulin secretion.

Acknowledgements

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