Intermediate Conductance Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels Modulate Human Placental Trophoblast Syncytialization

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Abstract

Regulation of human placental syncytiotrophoblast renewal by cytotrophoblast migration, aggregation/fusion and differentiation is essential for successful pregnancy. In several tissues, these events are regulated by intermediate conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (IK\textsubscript{Ca}), in part through their ability to regulate cell volume. We used cytotrophoblasts in primary culture to test the hypotheses that IK\textsubscript{Ca} participate in the formation of multinucleated syncytiotrophoblast and in syncytiotrophoblast volume homeostasis. Cytotrophoblasts were isolated from normal term placentas and cultured for 66 h. This preparation recreates syncytiotrophoblast formation in vivo, as mononucleate cells (15 h) fuse into multinucleate syncyntia (66 h) concomitant with elevated secretion of human chorionic gonadotropin (hCG). Cells were treated with the IK\textsubscript{Ca} inhibitor TRAM-34 (10 \textmu M) or activator DCEBIO (100 \textmu M). Culture medium was collected to measure hCG secretion and cells fixed for immunofluorescence with anti-IK\textsubscript{Ca} and anti-desmoplakin antibodies to assess IK\textsubscript{Ca} expression and multinucleation respectively. K\textsuperscript{+} channel activity was assessed by measuring \textsuperscript{86}Rb efflux at 66 h. IK\textsubscript{Ca} immunostaining was evident in nucleuses, cytoplasm and surface of mono- and multinucleate cells. DCEBIO increased \textsuperscript{86}Rb efflux 8.3-fold above control and this was inhibited by TRAM-34 (85% (\textit{p}<0.0001). Cytotrophoblast multinucleation increased 12-fold (\textit{p}<0.005) and hCG secretion 20-fold (\textit{p}<0.005), between 15 and 66 h. Compared to controls, DCEBIO reduced multinucleation by 42% (\textit{p}<0.005) and hCG secretion by 80% (\textit{p}<0.005). TRAM-34 alone did not affect cytotrophoblast multinucleation or hCG secretion. Hyposmotic solution increased \textsuperscript{86}Rb efflux 3.8-fold (\textit{p}<0.0001). This effect was dependent on extracellular Ca\textsuperscript{2+}, inhibited by TRAM-34 and 100 nM charybdotoxin (85% (\textit{p}<0.0001) and 43% respectively) but unaffected by 100 mM apamin. In conclusion, IK\textsubscript{Ca} are expressed in cytotrophoblasts and their activation inhibits the formation of multinucleated cells in vitro. IK\textsubscript{Ca} are stimulated by syncytiotrophoblast swelling implicating a role in syncytiotrophoblast volume homeostasis. Inappropriate activation of IK\textsubscript{Ca} in pathophysiological conditions could compromise syncytiotrophoblast turnover and volume homeostasis in pregnancy disease.

Introduction

The syncytiotrophoblast is the transporting epithelium of the human placenta being the interface between maternal and fetal blood. This highly specialized epithelial cell also performs a number of other functions including hormone production and secretion. Syncytiotrophoblast has a short life span and is renewed by cellular turnover in a tightly regulated process where proliferative mononucleate cytotrophoblasts exit the cell cycle, differentiate and fuse with the overlying syncytial layer [1]; both apoptosis and autophagy have been hypothesized to play a role in completing turnover [2,3].

In \textit{vitro} models have been used to study some of the features of syncytiotrophoblast turnover. These include cytotrophoblasts isolated from normal term placenta and maintained in primary culture [4,5]. After 15–18 h of culture, cytotrophoblasts are predominantly mononucleate and secrete small amounts of human chorionic gonadotropin (hCG). Over 24–66 h they migrate, aggregate and fuse to become multinucleated, a process reminis-
Cytotrophoblasts isolated from placentas of women with pre-eclampsia have a lower rate of syncytialization than those of normal pregnancy [22]. Expression of syncytin-1 [23] and syncytin-2 [24], envelope fusogenic proteins that induce syncytiotrophoblast formation [23,25,26], is downregulated both in isolated cytotrophoblasts and placent al villous tissue from pregnancies complicated with pre-eclampsia [22,24,27,28]. Syncytiotrophoblast expression of other fusogenic proteins, for example e-cadherin [16], is also reduced in pre-eclampsia. Collectively, dysregulation of the processes contributing to syncytiotrophoblast renewal culminates in a decrease in the total volume of syncytiotrophoblast in pregnancies complicated by pre-eclampsia and fetal growth restriction [29]. This has implications for nutrient delivery to the fetus as syncytiotrophoblast volume correlates with fetal weight [30]. However, the intracellular and extracellular signals that trigger and regulate cytotrophoblast fusion to form syncytiotrophoblast are not well understood.

In non-placental tissues, cellular proliferation, fusion and apoptosis can be regulated by members of the Ca²⁺-activated K⁺ channel (IKCa) family, in particular by intermediate conductance Ca²⁺-activated K⁺ channels (IKCa; KCa3.1; single channel conductance 50–200 pS). IKCa are voltage-insensitive and are strongly activated by increased concentrations of intracellular Ca²⁺ ([Ca²⁺]]; 300–700 nM) [31,32]. IKCa mRNA was shown to be highly expressed by human placenta over 15 years ago [33] but the functions of IKCa in the placenta have not been explored.

A major function of IKCa is to regulate cellular volume [34–38]. IKCa activation induces K⁺ efflux from cells, which both lowers intracellular K⁺ concentration and promotes the loss of water by osmosis to induce cell shrinkage [39]. Appropriate adjustment of cell volume and/or intracellular K⁺ concentration is essential for cells to undergo proliferation, migration, fusion and apoptosis [40]. Indeed, in non-placental tissues, IKCa has been shown to contribute to tissue homeostasis by regulating proliferation [31,41–43], differentiation/fusion [44,45], cell migration [46–48] and apoptosis [49]. The ability of IKCa to regulate cell volume has been revealed experimentally by exposing cells to an osmotic challenge [34,35,37,50]. When placed in hyposmotic solutions, cells initially swell but then restore their volume by a process of regulatory volume decrease (RVD). In many cells hypsomotic cell swelling elevates intracellular Ca²⁺ which activates IKCa to promote K⁺ efflux and water follows to achieve RVD [34]. However, a role for IKCa in regulating renewal of syncytiotrophoblast and/or syncytiotrophoblast volume has yet to be explored.

We tested the hypotheses that IKCa participates in the formation of multinucleate syncytiotrophoblast and that IKCa has a role in syncytiotrophoblast volume regulation. Using isolated cytoto rhoblasts in primary culture we confirmed IKCa protein expression and tested the effects of IKCa modulators on efflux, the formation of multinucleate syncytia and the secretion of hCG. To investigate whether IKCa participate in syncytiotrophoblast RVD, cells were exposed to hypsomotic solutions and efflux measured in the presence and absence of IKCa modulators.

Materials and Methods

Materials

Unless otherwise stated, all chemicals were from Sigma-Aldrich (Poole, UK).

Ethics Statement

Human placentas used in this study were obtained from St. Mary’s Hospital Maternity Unit (Manchester, UK) following written informed consent as approved by the Local Research Ethics Committee (North West (Haydock Park) Research Ethics Committee (Ref: 08/H1010/55)). Placentas were collected at term (37–42 weeks) following uncomplicated pregnancy and delivery of a healthy baby by vaginal or Caesarean section. Exclusion criteria were body mass index >30 (measured at booking), pregnancy hypertension/pre-eclampsia, fetal growth restriction, gestational diabetes. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Cytotrophoblast Isolation

Cytotrophoblasts were isolated from normal term placentas and cultured for 66 h. This is a well-characterized method [4,5,31–54] which recreates syncytiotrophoblast formation in vivo, as mononucleate cells (15 h) fuse into multinucleate syncytiotrophoblast (66 h) concomitantly with elevated secretion of hCG.

Cytotrophoblasts were obtained using an adaptation of the method used by Kliman et al. [5], as previously described [4]. Briefly, full thickness placenta samples (~2 cm³) were taken within 30 min of delivery and placed into sterile saline. Placent al villous tissue was further dissected from each sample after removal of the chorionic plate and decidua, ~30 g of villous tissue were obtained and submitted to digestion 3 times in Hank’s balanced salt solution containing 2.5% trypsin and 0.2 mg/ml deoxyribonuclease (DNase I) for 30 min at 37°C in agitation. After each digestion, 100 ml of supernatant were obtained, layered onto 5 ml newborn calf serum and spun for 10 min at 2200 rpm (1000 xg) at 20°C. Afterwards, pellets were resuspended in 1 ml Dulbecco’s modified Earle’s medium (DMEM; Invitrogen, Paisley, UK) and centrifuged for 10 min at 2200 rpm. The supernatant was discarded and the pellet resuspended in 6 ml DMEM and layered onto a discontinuous Percoll density gradient and centrifuged for 30 min at 2800 rpm (1500 xg). The bands between 35–55% Percoll were obtained and mixed with cell culture medium (DMEM: Ham’s F-12 Nutrient Mixture (Invitrogen, Paisley, UK) 1:1, 10% fetal calf serum (heat inactivated), 1% gentamicin, 0.2% benzylpenicillin, 0.2% streptomycin, 0.6% glutamine), before centrifugation at 2200 rpm for 10 min. The final pellet was resuspended in 2 ml of cell culture medium. Cells were plated onto 35 mm culture dishes (Nunc, Fisher Scientific, Loughborough, UK) in cell culture medium, or 16 mm coverslips in 12-well culture plates, at densities of 1–1.3 x 10⁶/ml and 1 x 10⁶/ml respectively at 37°C in a humidified incubator (95% air/5% CO₂).

Cytotrophoblast Primary Culture and Treatment

Cytotrophoblasts plated onto 16 mm coverslips were cultured for 66 h. Cultures were washed 3 times with phosphate-buffered saline (PBS) and cell culture medium was replaced with fresh medium at 15 and 42 h. Cells were untreated (control) or treated at 3, 15 and 42 h with IKCa modulators 100 μM DCEBIO (5, 6-dichloro-1-ethyl-1, 3-dihydro-2H-benzimidazol-2-one; IKCa activator) or 10 μM TRAM-34 (1-[2-chlorophenyl] diphenylmethyl)-1H-pyrazole; IKCa inhibitor). In both cases, the final concentration of dimethyl sulfoxide (DMSO) in the cell culture medium was 0.1%. Previous studies from this laboratory have shown that DMSO at 0.1% does not alter cytotrophoblast morphological or biochemical differentiation [51].
In addition, at 15, 42 and 66 h of culture, cells were fixed in absolute methanol (permeabilizing fixative; to detect intracellular immunostaining) for 20 min at −20°C or in 4% paraformaldehyde (PFA; non-permeabilizing fixative; to detect immunostaining associated with cellular surface) for 15 min at room temperature and stored in PBS at 4°C prior to immunofluorescence staining.

Measurement of Cytotrophoblast hCG Secretion

The β-subunit of hCG is secreted by terminally differentiated syncytiotrophoblast and was used as an indicator of cytotrophoblast differentiation in culture [51]. β-hCG was assayed in cell-conditioned culture medium at 15, 42 and 66 h of culture by ELISA (DRG Diagnostics, Marburg, Germany). Thawed samples were used following the instructions of the manufacturer. Optical density was measured at 450 nm using a VersaMax microplate reader (Molecular Devices, CA, USA). hCG secretion was expressed as mIU/ml/mg protein.

Immunofluorescent Staining

Methanol and PFA-fixed cells on 16 mm coverslips were washed in tris-buffered saline (TBS). Block of non-specific binding was performed for 30 min with 4% bovine serum albumin (BSA) in TBS. Cells were incubated for 1 h at room temperature with mouse monoclonal antibody to desmoplakin I+II (clone 2Q400; Abcam, Cambridge, UK) diluted 1:100 in TBS or mouse monoclonal antibody to IKCa (KCa3.1; clone 6C1; extracellular epitope; Alomone labs, Jerusalem, Israel), diluted 1:50 in 1% BSA in TBS. Negative control was obtained by omission of the primary antibody. Cells were washed with TBS and the secondary antibody, FITC-polyclonal rabbit anti-mouse immunoglobulin (Dako, Cambridgeshire, UK) diluted 1:50 in TBS, was applied and cells incubated for 1 h at room temperature in the dark. After washing with TBS, coverslips were mounted using Vectashield mounting medium with propidium iodide nuclear counterstain (PI; Vector labs, Peterborough, UK). Immunofluorescent images were captured using a Zeiss AxiosObserver Inverted Microscope (magnification 400×).

Analysis of Cytotrophoblast Multinucleation

Microscope images of cytotrophoblasts stained for desmoplakin and nuclei were used to assess multinucleation as a measurement of cytotrophoblast morphological differentiation. Based on a previously published method [51,55], 2–3 observers counted the total number of nuclei per given field and the number of nuclei in syncytiotrophoblast and was used as an indicator of cytotrophoblast differentiation in culture [51]. β-hCG was assayed in cell-conditioned culture medium at 15, 42 and 66 h of culture by ELISA (DRG Diagnostics, Marburg, Germany). Thawed samples were used following the instructions of the manufacturer. Optical density was measured at 450 nm using a VersaMax microplate reader (Molecular Devices, CA, USA). hCG secretion was expressed as mIU/ml/mg protein.

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86Rb Efflux from Cytotrophoblasts

86Rb is commonly used as a tracer of K+ and it has been previously shown that K+ channels are permeable to 86Rb [33]. 86Rb efflux was measured in cytotrophoblasts at 66 h of culture using a technique previously described [33]. Briefly, cells plated onto 35 mm dishes were removed from the incubator and washed in control Tyrode’s buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5.6 mM glucose; pH 7.4; osmolality ~283 mOsm/kgH2O; isotonic compared to maternal plasma at term [56]; osmolality measured by freezing point depression). Cells were incubated with 1 ml 4 Ci/ml 86Rb (89.7 μM; concentration 1 μCi/ml; stock activity 1 mCi) for 2 h at room temperature. After washing for 3 min in 2 × 25 ml Tyrode’s buffer (with no added isotope), 86Rb efflux was measured by the sequential addition and removal of 1 ml Tyrode’s buffer at 1 min intervals; samples were collected every 1 min over 15 min (control, basal 86Rb efflux) and/or exposed to various treatments over 5–15 min (experimental period): 10 μM TRAM-34, 100 μM DCEBIO, 100 mM aminap (small conductance Ca2+-activated K+ channel (SKCa) inhibitor), 100 mM charybdotoxin (ChTx; IKCa (KCa3.1) inhibitor), 100 mM apamin (large conductance Ca2+-activated K+ channel (BKCa) inhibitor), 55 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5.6 mM glucose; pH 7.4; osmolality 145 mOsm/kgH2O; Ca2+-free hyposmotic solution (extracellular Ca2+ was buffered by removing CaCl2 and adding 0.5 mM EGTA). When used together, a pre-block-inhibition with TRAM-34 was performed at min 4 before adding DCEBIO-TRAM-34 or hyposmotic solution-TRAM-34. In a different set of experiments, 86Rb efflux was measured in efflux buffer with osmolality ranging 283–138 mOsm/kgH2O, which was obtained by varying the NaCl concentration.

After 15 min, the cells were lysed in 0.3 M NaOH for ~1 h and scraped in order to release intracellular 86Rb which was then counted in the supernatant to give a measure of total 86Rb remaining in the cells at the end of the experiment (cellular 86Rb). Effluxed and cellular 86Rb was measured in a gamma-counter (Packard Cobra II Auto Gamma, CA, USA). All counts recorded were at least 10 times higher than background counts.

The time course of percentage (%) 86Rb efflux was calculated at each time point as [(86Rb effluxed/86Rb in cells) x100]. The efflux rate constant was also determined making the assumption that 86Rb efflux at steady state reflects the loss of 86Rb from a single compartment (syncytiotrophoblast) limited by the K+ permeability of the plasma membrane. Consequently, the loss of 86Rb was measured by a first-order rate constant which was calculated over 10 min experimental period as (86Rb in cell at time t/86Rb in cell at time 0) where t0 is the cellular 86Rb at the start of the experiment.

Expression of Results and Statistics

Statistical analysis was performed using GraphPad Prism version 5 software. hCG secretion and multinucleation from control untreated cytotrophoblasts was expressed as mean ± standard error (SE) with n as the number of placentas. hCG secretion and multinucleation in TRAM-34 and DCEBIO-treated cells was expressed as median ± interquartile range (IQR), and analyzed with Friedman’s test with Dunn’s post hoc test. The relationship between 86Rb efflux and extracellular fluid osmolality was analyzed comparing control vs. each experimental osmolality using ANOVA with Turkey Kramer multicomparison post hoc test. Each value was expressed as mean ± SE. %86Rb efflux from multinucleated cytotrophoblasts was expressed as mean ± SE for each time point. The effects of all treatments on 86Rb efflux were assessed for statistical significance by comparing the differences in the slopes and intercepts of the rate constants using least squares linear regression analysis. In all cases, a p value less than 0.05 was considered statistically significant.

Results

Expression of IKCa in Cytotrophoblasts

IKCa protein expression was confirmed in mono (Figure 1A) and multinucleated (Figure 1B, C) cytotrophoblasts using immunofluorescent staining with a specific antibody which detects an extracellular site in the pore forming domain (S5–6) of human IKCa (KCa3.1). IKCa immunostaining was detected in cells fixed...
with methanol (intracellular staining; Figures 1A, B) or with PFA (associated with cytotrophoblast surface; Figure 1C).

At 15 h, IKCa staining (green) was evident in the nucleus (red; nuclear counterstain) of mononucleate cells, but also in the cytoplasm and surface of cell aggregates (Figure 1A). At 66 h, IKCa was associated to both the cytoplasm (Figure 1B) and cell surface (Figure 1C) of multinucleated cytotrophoblasts. Arrows indicate specific areas were the staining was associated to the cell surface. Figure 1D corresponds to a representative negative control showing that non-specific staining was not observed.

Functional expression of IKCa was confirmed by measuring 86Rb efflux, an indirect assessment of K+ permeability, in
multinucleated cytotrophoblasts after 66 h of culture. The time course of % 86Rb efflux/min is plotted in Figure 1E and F. Basal % 86Rb efflux in control cytotrophoblasts showed a stable steady state over 15 min (Figure 1E; black circles). DCEBIO, an IKCa activator, caused a marked rapid increase (8.3-fold) in 86Rb efflux which was completely blocked by TRAM-34 (85%), an IKCa inhibitor (Figure 1E). Rate constants, taken as the slopes of the regression lines fitted over the experimental period (10 min), were calculated and for all treatments the data could be fitted by a single exponential (Table 1). The fall in intracellular 86Rb (slope) was significantly greater with DCEBIO compared to DCEBIO + TRAM-34 and controls. TRAM-34 had no effect on basal 86Rb efflux (Figure 1F). The increase in 86Rb efflux with DCEBIO confirms the functional expression of IKCa in multinucleated cytotrophoblasts.

**Differentiation of Cytotrophoblasts in Culture**

We confirmed previous reports of cytotrophoblast morphological and biochemical differentiation in culture [4,5,51]. Figures 2A-C show representative phase contrast images depicting cytotrophoblast morphology. The arrows indicate mononuclear cells at 15 h, aggregates at 42 h and multinucleate cytotrophoblasts at 66 h (in Figures 2A, 2B and 2C respectively). Desmoplakin immunostaining (Figures 2D-F) confirmed this progression of morphological differentiation and was used to calculate the % of nuclei in multinucleate cells (multinucleation) at 15, 42 and 66 h of culture. At 15 h, cytotrophoblasts remained mononuclear (Figure 2D), at 42 h the cells had aggregated (Figure 2E) and at 66 h, cytotrophoblasts had fused to become multinucleated as indicated by the absence of desmoplakin staining (≥3 nuclei in syncytiotrophoblasts; Figure 2F). Cytotrophoblast multinucleation increased 12-fold between 15 and 66 h (Figure 2G). This morphological progression was accompanied by biochemical differentiation as indicated by an increase in hCG secretion (Figure 2H). Cytotrophoblast β-hCG secretion increased 20-fold between 15 and 66 h (Figure 2H).

**Effect of IKCa Modulators on Cytotrophoblast Differentiation**

Cytotrophoblasts were treated at 3, 15 and 42 h of culture with IKCa modulators TRAM-34 and DCEBIO and multinucleation (% of nuclei in multinucleate cells) was assessed to determine morphological differentiation. Figures 3A–F show representative images of desmoplakin immunostaining (green) and PI (red; nuclei) in cytotrophoblasts at 15 (Figure 3A: control untreated, 3C: TRAM-34, 3E: DCEBIO-treated) and 66 h (Figure 3B: control, 3D: TRAM-34, 3F: DCEBIO-treated) of culture. Compared to controls, activation of IKCa with DCEBIO significantly reduced multinucleation by 42% (median ± IQR: 26.6 16.2/30.0 compared to 13.8 8.6/17.2 respectively) at 66 h of culture (Figure 3G). Multinucleation was unaffected by TRAM-34 (Figures 3C, 3D, G).

**Effect of IKCa Modulators on Cytotrophoblast hCG Secretion**

Compared to controls at 66 h, DCEBIO reduced β-hCG secretion by 80% (19.5 7.1/19.5; Figure 4A). This inhibition of differentiation was not associated with a fall in total cell protein (Figure 4B), a proxy measure of cell number, suggesting that DCEBIO did not have a generalized toxic effect. On the contrary, DCEBIO caused a transient increase in cell protein at 42 h (148.8 134.8/157.1; Figure 4B). TRAM-34 did not affect cytotrophoblast hCG secretion (Figure 4A) or total cell protein (Figure 4B). In addition, the total number of nuclei was unaffected by the treatment with TRAM-34; however, treatment with DCEBIO caused a transient increase in the total number of nuclei at 15 h of culture (Figure 4C).

**Effect of IKCa Inhibitor on Swelling-activated K⁺ Efflux from Cytotrophoblasts**

A role for IKCa in regulating syncytiotrophoblast volume was explored using multinucleated cytotrophoblasts. We investigated the participation of IKCa in syncytiotrophoblast RVD by experimentally exposing cytotrophoblasts to a hypsometric solution and measuring 86Rb efflux as a marker of syncytiotrophoblast K⁺ permeability.

Figure 5A shows the relationship between 86Rb efflux and extracellular fluid osmolality (ranging from 283–138 mOsm/kgH₂O). Total 86Rb efflux over 10 min (experimental period) was plotted against the reciprocal value for the osmolality of the fluid bathing the cytotrophoblasts after 66 h of culture. A reduction in osmolality to 218 mOsm/kgH₂O (77% of control),

### Table 1. Mean rate constants of 86Rb efflux in control and treated cytotrophoblasts.

| Condition                  | 86Rb efflux rate constant (l, 86Rb in cell (t=x)/(t=0))/min⁻¹ | r²         | p value | n |
|----------------------------|---------------------------------------------------------------|------------|---------|---|
| Control                    | −0.015±0.001                                                 | 0.660      | –       | 8 |
| Control-100 μM DCEBIO      | −0.068±0.005*                                                | 0.863      | <0.0001 | 3 |
| Control-100 μM DCEBIO+10 μM TRAM-34 | −0.013±0.004                                                | 0.284      | 0.689   | 3 |
| Control-10 μM TRAM-34      | −0.014±0.001**                                               | 0.861      | 0.985   | 4 |
| Hyposomatic solution       | −0.032±0.001***                                              | 0.933      | <0.0001 | 6 |
| Hyposomatic solution+100 mM apamin | −0.036±0.003                                               | 0.854      | 0.799   | 3 |
| Hyposomatic solution+100 mM ChTx | −0.018±0.001†                                               | 0.885      | <0.0001 | 3 |
| Hyposomatic solution+10 μM TRAM-34 | −0.013±0.001†                                              | 0.867      | <0.0001 | 5 |

Data are mean ± SE, n is the number of placentas. p values determined by linear regression:

*compared to corresponding control (−0.011±0.002/min⁻¹; r² 0.628) and 100 μM DCEBIO+10 μM TRAM-34 (−0.013±0.004/min⁻¹; r² 0.284);
**compared to control (−0.014±0.001/min⁻¹; r² 0.823);
***compared to control (−0.013±0.001/min⁻¹; r² 0.763);
†compared to hyposomatic solution (−0.035±0.003/min⁻¹; r² 0.808);
‡compared to hypotonic solution (−0.032±0.001/min⁻¹; r² 0.925).

doi:10.1371/journal.pone.0090961.t001
stimulated $^{86}$Rb efflux compared to control (283 mOsm/kgH$_2$O). Reducing extracellular osmolality to 183 and 138 mOsm/kgH$_2$O (65 and 49% of control respectively) progressively stimulated $^{86}$Rb efflux over control. Consequently, the minimum extracellular osmolality required to trigger $^{86}$Rb efflux from multinucleated cytotrophoblasts is between 77–65% isotonic. Therefore, the remaining experiments were performed using a hyposmotic solution with an osmolality of 145 mOsm/kgH$_2$O.

In agreement with previous results in placental villous tissue [57], exposure of multinucleated cytotrophoblasts to a hyposmotic solution markedly increased $^{86}$Rb efflux (3.8-fold; Figure 5B). The rate constant (Table 1) for $^{86}$Rb efflux was significantly greater in cytotrophoblasts exposed to the hyposmotic solution than controls. In addition, swelling-activated $^{86}$Rb efflux was Ca$^{2+}$-dependent, as removal of Ca$^{2+}$ from the hyposmotic solution abolished the activation of $^{86}$Rb efflux at 66 h of culture (Figure 5B; Table 1).

Discussion

This study shows that IK$_{Ca}$ protein is expressed by mono- and multinucleate cytotrophoblasts in vitro. Multinucleate cells show...
Figure 3. DCEBIO reduces cytotrophoblast multinucleation. Representative dual immunofluorescent staining showing desmoplakin (green) and nuclear counterstain (red) in control untreated (A, B), TRAM-34 (C, D) or DCEBIO (E, F) treated cytotrophoblasts at 15 (A, C, E) and 66 h (B, D, F) of culture.
Cultures. Arrows in B indicate multinucleated cytotrophoblasts at 66 h of culture. Scale bar 50 μm. G: The % of cytotrophoblast nuclei in multinucleate cells (multinucleation) at 15, 42 and 66 h of culture (n = 6 placentas); *p<0.05; Friedman’s test with Dunn’s post hoc test. Data are median ± IQR.

doi:10.1371/journal.pone.0090961.g003

Figure 4. DCEBIO inhibits cytotrophoblast hCG secretion. (A) β-hCG secretion, (B) cell protein and (C) total number of nuclei in cytotrophoblasts at 15, 42 and 66 h of culture in controls and cells treated with TRAM-34 or DCEBIO; n = 6 placentas; *p<0.05; Friedman’s test with Dunn’s post hoc test. Data are median ± IQR.

doi:10.1371/journal.pone.0090961.g004
low inherent IKCa activity as TRAM-34, an inhibitor of IKCa did not alter basal 86Rb efflux. However, DCEBIO stimulated TRAM-34-sensitive 86Rb efflux from multinucleate cytotrophoblasts (66 h). Time course of 86Rb efflux over 13 min during the experimental period (indicated by the bar) cells were (B) untreated (control) or exposed to hyposmotic solution (145 mOsm/kgH2O) or Ca2+-free hyposmotic (n = 6 placentas); (C) control, hyposmotic solution or hyposmotic solution + TRAM-34 (n = 5 placentas); (D) hyposmotic solution, hyposmotic solution + apamin or hyposmotic solution + charybdotoxin (ChTx) (n = 5 placentas). Data are mean ± SE.

doi:10.1371/journal.pone.0090961.g005

IKCa Expression and Function in Cytotrophoblasts from Term Placentas

Immunofluorescent staining of cytotrophoblasts confirmed the expression of IKCa protein in mononuclear, aggregated and multinucleated cells. IKCa staining was associated with the nucleus, cytoplasm and cytotrophoblast cell surface regardless of differentiation stage. Other K+ channels, such as Kv8 [58] and KCa8 [59,60] have been localized to the cell nucleus in various cell types; it has been suggested that KCa8 could control Ca2+ release and mobilization within the cell nucleus [59]. In addition, there is evidence of intracellular localization of KCa8 which may be associated with different cellular functions in non-placental cell types, e.g. in mitochondria [61], intracellular trafficking [62]. Therefore, the heterogeneous localization of IKCa could be related to diverse functions that these channels might have in cytotrophoblasts during differentiation.

The functional expression of IKCa was assessed using 86Rb efflux as a tracer of K+ efflux. The results indicate that multinucleated cytotrophoblasts express functional IKCa as exposure to the IKCa activator DCEBIO, significantly increased 86Rb efflux. DCEBIO was specific for IKCa since this increase in efflux was completely blocked by TRAM-34. However, in a quiescent state IKCa are inactive as TRAM-34 did not affect basal 86Rb efflux. This opens the possibility that different stimuli can activate IKCa in cytotrophoblasts under physiological/pathophysiological conditions but this remains to be determined.

Role of IKCa in Cytotrophoblast Multinucleation

Cytotrophoblasts isolated from term placentas subjected to trypsin-DNAse digestion and Percoll gradient separation are enriched in trophoblast markers and lack contamination from other placental cell types such as, endothelial cells, smooth muscle cells, fibroblasts, or macrophages [4,5]. After isolation and during the first hours, these cells, which are mitotically inactive, remain mononucleated and secrete small amounts of hCG. After 24 h in...
Role of IKCa in Syncytiotrophoblast Volume Regulation

In many cell types, restoration of cell volume in the presence of a hyposmotic stimulus (RVD) is mediated by K⁺ channels, including IKCa, in conjunction with swelling-activated anion channels [39]. In the current study, exposing multinucleated cytotrophoblasts to a hyposmotic solution increased ⁸⁶Rb efflux ~3.8-fold and this activated efflux was dependent on extracellular Ca²⁺, blocked (>80%) by the IKCa inhibitors TRAM-34 and ChTx but was unaffected by the SKCa inhibitor apamin. These data implicate IKCa in cytotrophoblast RVD. Lowering extracellular osmolality also stimulated ⁸⁶Rb efflux from placental villous tissue [57] and caused a Ba²⁺-sensitive hyperpolarization of the syncytiotrophoblast microvillous membrane [69]; however, the identities of the K⁺ channels underlying the resting conductance, or the change with cell swelling, remain unknown.

Exposing cells to a hyposmotic solution is an experimental maneuver often used to mimic the cell swelling which takes place secondary to a rise in intracellular osmolality as can occur following nutrient uptake [39,69,70]. In this case, activation of K⁺ channels is a homeostatic process to promote water loss to restore the concentration of cytoplasmic constituents and to shrink cells back to their original size. On the other hand, in the absence of hyposmotic swelling, the activation of K⁺ channels to promote water loss effects a cell volume change, and/or fall in intracellular K⁺, that is essential for a variety of processes that maintain tissue homeostasis such as cell proliferation, migration, differentiation/fusion and cell death [71]. It is possible that dynamic changes in cell volume are required for normal cytotrophoblast fusion and that, in the current study, chronically activating IKCa channels induced an inappropriate change in cell volume which inhibited fusion. Cytotrophoblast fusion may be altered by promoting IKCa activity and consequently inducing water loss which alters the concentration of cytoplasmic factors that regulate fusion. These proposals need to be investigated in future, and in particular elucidate whether the primary effect of activation of IKCa is on fusion.

Conclusions

The primary stimuli for IKCa activation is an elevation in [Ca²⁺]i and therefore factors that increase [Ca²⁺]i will activate cytotrophoblast IKCa. To date there are relatively few studies of the regulation of [Ca²⁺]i in syncytiotrophoblast; however, preliminary evidence indicates that hyposmotic swelling increases [Ca²⁺]i, in multinucleated cytotrophoblasts, predominantly by entry from extracellular fluid [72].

Consequently, activation of IKCa could regulate syncytiotrophoblast volume, which can change dynamically following solute uptake and/or cytotrophoblast cell fusion, an essential homeostatic mechanism to maintain nutrient transport and endocrine function respectively. In addition, we have previously shown that cytotrophoblast [Ca²⁺]i, is elevated following activation of purinergic receptors, including P2X₄, by extracellular nucleotides and that this promotes ⁸⁶Rb efflux which is inhibited by ChTx, implicating activation of IKCa. These findings might be of relevance to the etiology of pre-eclampsia, a disease of pregnancy characterized by abnormal cytotrophoblast fusion and renewal of syncytiotrophoblast. Indeed, the expression of P2X₄ by the placenta is elevated in pre-eclampsia compared to normal pregnancy [73]. It is also proposed that hypoxia/elevated reactive oxygen species release nucleotides from the trophoblast in pre-eclampsia to elevate local concentrations in the extracellular fluid [73,74]. As a result, increased activation of P2X₄ would elevate [Ca²⁺]i, and activate IKCa. The inappropriate activation of IKCa could compromise...
cell volume homeostasis, and impact on cytotrophoblast cell fusion and syncytiotrophoblast renewal, endocrine function and nutrient transport in pre-eclampsia.

Acknowledgments

The authors wish to thank the midwives and patients at the Maternity Unit at St. Mary’s Hospital, Manchester, UK, for their assistance.

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